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The Ess/Type VII secretion system of *Staphylococcus aureus* secretes a nuclease toxin that targets competitor bacteria

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Summary

The type VII protein secretion system (T7SS) plays a critical role in the virulence of human pathogens including *Mycobacterium tuberculosis* and *Staphylococcus aureus*. Here we report that the *S. aureus* T7SS secretes a large nuclease toxin, EsaD. The toxic activity of EsaD is neutralised during its biosynthesis through complex formation with an antitoxin, EsaG, which binds to its C-terminal nuclease domain. The secretion of EsaD is dependent upon a further accessory protein, EsaE, that does not interact with the nuclease domain, but instead binds to the EsaD N-terminal region. EsaE has a dual cytoplasmic/membrane localization and membrane-bound EsaE interacts with the T7SS secretion ATPase, EssC, implicating EsaE in targeting the EsaDG complex to the secretion apparatus. EsaD and EsaE are co-secreted whereas EsaG is found only in the cytoplasm and may be stripped off during the secretion process. Strain variants of *S. aureus* that lack *esaD* encode at least two copies of EsaG-like proteins most likely to protect themselves from the toxic activity of EsaD secreted by *esaD*$^+$ strains. In support of this, a strain overproducing EsaD elicits significant growth inhibition against a sensitive strain. We conclude that T7SSs may play unexpected and key roles in bacterial competitiveness.

Protein secretion systems are used by bacteria to interact with and manipulate their environments, and play critical roles in the secretion of virulence factors. Gram-negative bacteria produce numerous secretion systems that transport substrates across the cell envelope[1]. Many Gram-positive bacteria also produce a specialised protein secretion machinery termed the Type VII secretion system (T7SS). T7SSs are found in representatives of the Actinobacteria and Firmicutes phyla. The system was first described in the Actinobacterial pathogens *Mycobacterium tuberculosis* and *M. bovis* where T7SS ESX-1 was shown to be essential for virulence and to secrete two small proteins ESAT-6 and CFP-10, subsequently renamed EsxA and EsxB[2-4]. In addition to secreting EsxA/B proteins, Mycobacterial T7SSs can also secrete much larger proteins of the PE/PPE family[5], which are highly abundant in the genomes of some species[6].

A distantly related T7SS, termed T7b[7], is also found in Firmicutes such as *Bacillus subtilis*[8,9] and *Staphylococcus aureus*[10]. T7SSs share two common types of components: a membrane-bound hexameric ATPase of the FtsK/SpoIIIE protein family[11,12] and at least one EsxA/EsxB-related protein[11]. EsxA and EsxB are members of the WXG100 superfamily that form dimeric helical hairpins[13,14], and in Firmicutes EsxA is exported as a folded homo-dimer[14,15]. The *S. aureus* Ess system comprises six core components[10,16] (Figs 1A; S1). In addition to EssC, three further membrane proteins EsaA, EssA, EssB are essential for T7 secretion activity[16,17] along with the secreted protein EsxA[10] and the predicted cytoplasmic protein EsaB[16]. All except one strain of *S. aureus* examined to date encode the six core T7 components but there is strain variability in the repertoire of T7 substrate proteins[18]. Studies using strains with the NCTC8325 T7S gene cluster organisation, including Newman, USA300 and RN6390 have identified EsxB, EsxC and EsxD as secreted substrates[10,16,19,20]. These three proteins are small (~100-130 aa), and their precise functions remain to be elucidated.

The T7SS has previously been shown to contribute to virulence in mouse infection models[10,16,21] and to facilitate release of intracellular *S. aureus* from epithelial cells[22]. Here we identify a further function for the *S. aureus* T7SS in bacterial competition. We show that EsaD is a T7 nuclease substrate that interacts with two Ess accessory proteins, EsaG, an antitoxin, and EsaE, a putative chaperone, during its biosynthesis. Strains of *S. aureus* that do not encode the EsaD substrate harbour *esaG* homologues, most likely to protect themselves from killing by EsaD-producing strains. In support of this we demonstrate EsaD-dependent growth inhibition of *S. aureus*. Our findings confirm that the Gram-positive T7SS has anti-bacterial activity in addition to anti-eukaryotic function.

Results

EsaD is not required for T7SS activity. EsaD is encoded within the T7SS gene cluster (Fig 1a) and has been reported to be a membrane-bound T7SS accessory factor[23], however its absence from some *S. aureus* strains indicates it is unlikely to be a critical component of the secretion machinery[18]. We constructed an in-frame *esaD* deletion and asked whether it was

required for secretion of the core component EsxA and the substrate protein EsxC. Fig 1b shows that EsxA was still secreted by the *esaD* strain, but very little EsxC was detected. To circumvent this we overproduced EsxC from a plasmid (Fig 1c), and in this case could clearly detect EsxC in culture supernatants of the *esaD* strain. We conclude that EsaD is not essential for T7-dependent secretion.

EsaD is a predicted nuclease that is secreted by the T7SS. EsaD encoded by strain NCTC8325 is predicted to be a 614 residue protein, and sequence analysis suggests that the C-terminal ~170 aa comprise a nuclease domain (Fig S2). In accord with EsaD having toxic activity, we were unable to clone *esaD* unless we introduced a H528A codon substitution at the predicted nuclease active site. When HA-tagged EsaD(H528A) was produced in either *S. aureus* RN6390 or COL strains, tagged protein was detected in the supernatant (Fig 1d, e). The lack of EsaD in supernatants of the cognate *essC* strains strongly suggests that EsaD is a T7SS secreted substrate.

EsaD has previously been reported as a membrane protein[23]. However, topology prediction programmes (e.g. TMHMM) do not predict transmembrane regions. To explore the location of cellular EsaD we fractionated cells producing EsaD(H528A)-HA and Fig 1f shows the tagged protein (migrating as a double band) was clearly detected only in the cytoplasmic fraction. We conclude EsaD is not a membrane protein.

EsaD has toxic activity that is neutralised by EsaG. To confirm that EsaD is a toxin, it was essential to clone the wild-type gene. We could readily clone *esaD* if the downstream gene, *esaG*, was included, but not if this sequence was omitted. We were eventually able to obtain a clone of *esaD* in pT7.5[24]. Expression of genes from this vector is under control of the T7 promoter which is not recognised by *E. coli* RNA polymerase. Even so, the clone we obtained, which gave very small colonies, harboured a V584Y substitution. We reasoned that this might serve to lower the stability/toxicity of EsaD. When this construct was introduced into *E. coli* BL21(DE3) (that encodes an inducible copy of the phage T7 RNA polymerase), growth ceased when the inducer, IPTG was added (Fig 2a) but cells continued to grow if *esaG* was co-

expressed with wild type *esaD*. We conclude that EsaD has toxic activity that is modulated by EsaG.

Examination of *E. coli* cells by microscopy showed that production of EsaD(V584Y) resulted in cell elongation (Fig S3a,b), a hallmark of the SOS response induced by DNA damage[25,26], consistent with EsaD exhibiting DNase activity. We confirmed EsaD-induced DNA damage using TUNEL, which labels the ends of fragmented DNA[27,28]. Fluorescence microscopy showed TUNEL staining in a subset of cells producing EsaD(V584Y) (Fig S3c) that could also be detected by flow cytometry (red dots in FACS plot in Fig S3d). Production of EsaD was also associated with an increase in side scatter, consistent with changes in cellular morphology seen by microscopy. We conclude that EsaD results in DNA damage when produced in the cytoplasm of *E. coli*.

EsaG interacts with the nuclease domain of EsaD. The presence of *esaG* counteracts the toxic activity of EsaD suggesting that *esaG* is an antitoxic gene. Antitoxins may be proteins or RNA[29]. Inspection of *esaG* indicates that it is a probable protein-coding gene, producing a protein of the uncharacterised DUF600 family. To investigate whether EsaG interacts with EsaD we co-produced EsaD(H528A)-His with EsaG-HA in *S. aureus* and purified tagged EsaD from cell lysates. Fig 2c shows that EsaG co-purifies with EsaD indicating the proteins form a complex.

EsaD-EsaG interaction was also confirmed by bacterial two hybrid assay (Fig 2b). EsaG (163 aa) is significantly smaller than EsaD, suggesting it may interact with only part of EsaD. We genetically separated EsaD into predicted nuclease domain ($EsaD_{421-614}$, harbouring the H528A codon substitution) and N-terminal region ($EsaD_{1-420}$). Fig 2b shows that EsaG interacts specifically with the predicted nuclease domain. This was confirmed biochemically by co-purification of EsaG-HA with His-tagged $EsaD_{421-614}$ (Fig 2d). We conclude that EsaG is a proteinaceous antitoxin that blocks EsaD activity by direct interaction.

To investigate directly whether EsaD has DNase activity, we overproduced His-tagged wild type or H528A variants of the nuclease domain in *E. coli* in the presence of EsaG and purified His-tagged EsaD in the presence of 8M urea to unfold the protein and detach bound EsaG

(Fig 2e). After refolding and eluting from the Ni-resin, the EsaD nuclease domains were incubated with plasmid DNA in the presence of Mg^{2+} or Zn^{2+} ions. Fig 2f shows that wild type EsaD specifically degraded plasmid DNA in the presence of Mg^{2+},and could also degrade genomic and linear DNA (Fig S5). The H528A variant showed some DNAse activity, but appeared to be much less potent than EsaD, as expected. We conclude that EsaD is a Mg^{2+}-dependent DNase.

Fractionation of cells overproducing tagged EsaG shows that it is found exclusively in the cytoplasm (Fig S6). However, as the T7SS can export protein complexes[15], we tested whether it could be co-secreted if overproduced with EsaD. Fig S7 shows that in the presence of secreted EsaD(H528A), EsaG localised only to the cell fraction and does not appear to be co-secreted with its partner protein. We then asked whether EsaG was required for secretion of EsaD. We were not able to delete *esaG* from *S. aureus* unless we also deleted *esaD*, consistent with its antitoxic role. Production of HA-tagged EsaD(H528A) in the *esaDG* mutant strain resulted in no detectable EsaD in the supernatant and very little in the cells (Fig S8). We conclude that EsaG is required for the stability/secretion of EsaD. EsaG was not, however, required for the secretion of either EsxA or EsxC (Fig S9) and is therefore not a general T7S accessory factor.

EsaE also interacts with EsaD and is required for its secretion or stability. Studies of Mycobacterial T7SS have revealed that PE/PPE substrate proteins interact with specific chaperones that facilitate their secretion[30-32]. To investigate whether any additional soluble proteins encoded at the *S. aureus* T7SS locus interact with EsaD, we used bacterial two hybrid analysis. Fig 3a shows no evidence for EsaD interaction with any known secretion substrates (EsxB, EsxC or EsxD) or soluble machinery components (EsaB or EsxA). Interaction was detected between EsaD and EsaE, which was also confirmed biochemically as EsaE-HA co-purified with EsaD(H528A)-His when the two proteins were co-produced in *S. aureus* (Fig 3b). To determine whether EsaE interacted with the nuclease domain of EsaD or elsewhere on the protein, we screened EsaE interaction with $EsaD_{1-420}$ or $EsaD_{421-614}$(H528A) (Fig 3c). The results show that EsaE specifically interacts with the non-nuclease region of EsaD. We tried

to confirm these genetic observations with co-purification experiments but were not able to stably produce the truncated $EsaD_{1-420}$.

We constructed an in-frame deletion of *esaE* to determine whether it was required for EsaD secretion. We were readily able to obtain the *esaE* mutant, consistent with EsaE not being an antitoxin but playing some other role in EsaD biosynthesis. Production of HA-tagged EsaD(H528A) in this strain resulted in no detectable EsaD in the supernatant and very little in the cells (Fig S8), suggesting EsaE is required for its stability or secretion. Although EsaE was not required for EsxA secretion, there was no apparent secretion of EsxC in the *esaE* mutant, even if EsxC was overproduced from a plasmid (Fig S9). Thus EsaE is required for efficient secretion of EsaD and at least one further T7SS substrate.

His-tagged EsaE was found almost exclusively in the cellular fraction, suggesting it is not, by itself, a T7SS substrate (Fig 3d). We did, however, routinely observe that overproduction of tagged EsaE led to a dramatic increase in the level of extracellular EsxA (Fig 3d), for reasons that are unclear. However, when EsaE-His was co-produced with EsaD(H528A)-HA, notable secretion of His-tagged EsaE could now be detected suggesting that these two proteins are co-exported as a complex.

EsaD/E/G form a ternary complex. We next assessed whether EsaD could form a ternary complex with EsaE and EsaG. Control experiments (Fig S10) showed no direct interaction between EsaE and EsaG by either two hybrid or co-purification experiments. When EsaD(H528A)-Myc, EsaE-HA and EsaG-His were co-produced in *E. coli* and EsaE-HA immunoprecipitated, EsaG-His and Myc-tagged EsaD were also co-precipitated, consistent with the three proteins forming a ternary complex (Fig 3f,g). Reciprocal experiments where His-tagged EsaG was isolated by Ni-affinity purification resulted in co-purification of EsaE-HA and EsaD-Myc (Fig S12). In this latter experiment, although some full length EsaD-Myc was detected, most of the protein was fragmented to the approximate size of the nuclease domain, consistent with the prior instability of $EsaD_{1-420}$ noted previously.

EsaE interacts with the multimeric form of EssC. Although EsaE is predicted to be soluble, subcellular fractionation showed a proportion of His-tagged EsaE localised to the membrane

and was stable to carbonate washing (Fig 4a). As EsaE has a dual cytoplasmic-membrane location, we wondered whether it may play a role in targeting EsaD/EsaG to the membrane-bound secretion machinery. We undertook formaldehyde crosslinking experiments in whole cells of *S. aureus* producing EsaE-His, isolated membranes and blotted for EsaE. Fig 4b shows several EsaE-His crosslinks, including a particularly strong crosslink migrating above 250 kD. It has previously been shown that the *S. aureus* T7 ATPase, EssC, forms a high molecular weight multimer[17,33]. To ascertain whether this high molecular weight crosslink also contained EssC, we repeated the crosslinking experiments in the wild type and *essC* mutant strains. Fig 4c shows that the EsaE-His-containing crosslink migrated with an apparently identical mass as the EssC-containing crosslink and moreover, no such crosslink was detected when *essC* was deleted. We conclude that the membrane-bound form of EsaE interacts with the multimeric form of EssC.

These results suggest a model for the biosynthesis and secretion of EsaD (Fig 4d), whereby the interaction of EsaG and EsaE with their respective binding domains on EsaD is essential for maintaining EsaD in a catalytically-inactive, secretion-competent conformation. Our findings support the idea that targeting of the protein complex to the T7 secretion machinery is by virtue of the interaction between EsaE and the assembled EssC multimer and that EsaG is stripped from the complex at some point during secretion and remains in the cytoplasm. Thus EsaD is released from the cell in a form that is immediately active, and the EsaG immunity protein remains in the producing cell where it may potentially serve further protective functions.

Secreted EsaD inhibits the growth of sensitive strains of *S. aureus*. We next addressed potential roles for EsaD. Gram-negative bacteria utilise a subset of their protein secretion systems to target toxins at bacterial competitors as well as at eukaryotic cells[34-38]. It was noted previously that although approximately 50% of *S. aureus* strains do not carry *esaD*, they encode at least two homologues of *esaG* close to their T7SS gene clusters[18] (Fig 5a) suggesting they may produce EsaG-type proteins as a protective mechanism to prevent killing by EsaD-producing strains. To probe this, we assessed whether EsaG homologues from non

208 *esaD*-containing strains MRSA252, ST398 and EMRSA15 could interact with EsaD(H528A)
209 using two hybrid analysis. At least one EsaG homologue from each strain was able to interact
210 with EsaD (Fig 5b), supporting the idea that orphan EsaG proteins serve to protect *S. aureus*
211 from EsaD nuclease toxins.
212 A common feature of bacterial toxins, particularly those involved in interspecies competition,
213 is that that they are polymorphic[39,40]. Comparison of EsaD sequences across *esaD*-encoding
214 *S. aureus* strains shows extensive sequence variability within the nuclease domain but away
215 from the predicted catalytic site (centred around H528; Fig S13). A likely explanation is that
216 substitutions in this region of EsaD alter affinity for a cognate EsaG antitoxin and render
217 normally resistant strains susceptible to attack by EsaD sequence variants. In this context it is
218 interesting to note a cluster of EsaG homologues are encoded directly downstream of *esaDG*
219 in *S. aureus esaD*-containing strains that is highly variable in number[18]. For example
220 NCTC8325 encodes five of these (Fig 5a), whereas COL encodes eleven. These genes are
221 not co-transcribed with *esaDG* in strain RN6390 (Fig S14), however analysis of RNA-seq data
222 from[18] indicated that transcripts from these genes are present under laboratory growth
223 conditions.
224 We could readily delete the cluster of *esaG*-like genes from RN6390 indicating they are not
225 required to neutralise EsaD, consistent with our prior conclusion that EsaG itself is the cognate
226 EsaD antitoxin. Loss of this *esaG*-like gene cluster, or indeed absence of all EsaG-encoding
227 proteins (including EsaG) also did not affect the secretion of EsxA or EsxC (Fig S9) confirming
228 these proteins are not essential components of the T7SS machinery. However, two hybrid
229 assay shows that they are able to interact with EsaD (Fig 5c), but that the interaction is not as
230 strong as that seen for EsaD-EsaG, raising the possibility that they serve to protect RN6390
231 from EsaD sequence variants produced by other strains of *S. aureus*.
232 Together, the results presented above strongly suggest that *S. aureus* uses its T7SS to
233 secrete a nuclease toxin that targets rival bacteria. To confirm this, we used *S. aureus* strain
234 COL, which shows the highest level of T7S activity in laboratory growth media[16] producing
235 plasmid-encoded EsaDG as attacker, and incubated it with variants of strain RN6390. Fig 5d

shows that there is an approximate two-log decrease in recovered prey cells when they are co-cultured with a T7SS$^+$ strain of COL compared with a T7SS mutant strain, demonstrating there is T7-dependent growth inhibition. Importantly, this is completely dependent upon the toxic activity of EsaD as COL producing the H528A variant of EsaD no longer exhibited detectable growth inhibition (Fig 5d). Finally, as expected, EsaG offered some protection against the inhibitory effect of secreted EsaD as the RN6390 wild type tended to be less susceptible to EsaD-dependent growth inhibition than a strain lacking EsaG homologues, and the protective effect was enhanced by overproduction of plasmid-encoded EsaG in the prey cells. We conclude that *S. aureus* can use its T7SS to target bacterial competitors.

Discussion

It is well established that T7SSs play critical roles in mammalian infection and virulence. Here we demonstrate an important novel role for the *S. aureus* T7SS in the secretion of a nuclease toxin, EsaD, involved in interspecies competition. Thus, akin to the Gram-negative Type VI secretion system[34,41], the T7SS appears to target eukaryotes and rival bacteria.

EsaD is the largest known substrate of the T7b system and the only one for which a function has been identified. We have shown that two T7 accessory factors are essential for the biogenesis of EsaD - EsaG that binds to and neutralises the toxic activity of the nuclease domain and EsaE that interacts with, and potentially stabilises, the N-terminal region. In this context it is interesting to note that large substrates of the Mycobacterial T7a system such as the PE/PPE proteins interact with specific chaperones of the EspG family that keep them in a secretion-competent state[32] and deliver them to the cognate secretion machinery[31]. Although there is no detectable sequence similarity between EspG proteins and EsaE, it is possible that the proteins have analogous functions in the two distantly related secretion machineries.

The presence of T7SSs in non-pathogenic organisms such as *Streptomyces coelicolor*[42] and *Bacillus subtilis*[8,9] has previously been noted. Here we offer a likely explanation for the

presence of this secretion system in environmental strains. We have demonstrated that secreted EsaD inhibits the growth of sensitive strains of *S. aureus*, indicating this nuclease toxin is used to target competitor bacteria. In support, strains of *S. aureus* lacking *esaD* encode at least two copies of the EsaG-like antitoxin, presumably as a protective mechanism. It is interesting to note that an EsaD homologue has been reported in *B. subtilis*[23] that likely has toxic activity[43], inferring that modulating bacterial competition is a conserved role for T7SSs.

It is currently not known how EsaD accesses the cytoplasm of target cells. There is no evidence that T7SSs form large extracellular needle-like structures that could deliver toxins directly into target cells, like to those seen for the Gram-negative Type III, IV and VI secretion systems[1]. Instead we suggest that EsaD is released into the environment where it binds to receptors on sensitive cells in a similar manner as bacteriocins and contact-dependent growth inhibition (CDI) toxins, as the first step in a cell entry pathway[44,45]. Further work will be required to dissect out the mechanism by which EsaD interacts with and traverses the cell envelope of *S. aureus*.

S. aureus is an important pathogen in polymicrobial human infections, for example infections of the skin and lung. Whether EsaD-mediated interspecies competition is critical for the establishment of virulence in animal and human infections and whether there are additional antibacterial toxins secreted by the *S. aureus* T7SS remain to be established.

Methods

Bacterial strains and growth conditions: Strains and plasmids used in this study are listed in Tables S1 and S2. *S. aureus* strains were grown in TSB medium at 37°C under vigorous agitation. Where required, chloramphenicol (Cm) at a final concentration of 10 µg/ml was added for plasmid selection. Anhydrotetracycline (ATC) was used as a selection during allelic gene replacement using the pIMAY system (1 µg/ml[46]) or for induction of target gene expression from the pRAB11 plasmid[47]; the concentrations used in each experiment are listed

in the appropriate figure legends). *Escherichia coli* was grown aerobically in Lysogeny broth (LB) at 37°C. If required, cultures were supplemented with ampicillin (Amp, 100 µg/ml), Kanamycin (Kan, 50µg/ml) or Cm (15 µg/ml) for plasmid selection. Induction of plasmid-encoded gene expression was achieved by addition of isopropyl-β-D-galactopyranoside (IPTG), as indicated in the text. Light microscopy was carried out using a Zeiss light/fluorescence microscope with a 100x oil objective and images captured using an AXIO camera (Zeiss). The light microscopy images in Fig S3a were performed twice using different biological samples, representative images are shown. Bacterial two-hybrid analyses were performed as described[48]; quantitative assessment of protein interactions was undertaken by plating onto MacConkey medium[49] containing 0.4% maltose as carbon source, and quantified by β-galactosidase assays (according to the method of[50]) on strains grown to exponential phase at 30°C and permeabilized with toluene. For all of the bacterial two hybrid experiments reported (Figs 2b, 3a, 3c, 5b, 5c, S10a) each interaction pair was scored on MacConkey maltose on at least four different occasions and β-galactosidase assays were performed at least twice, and representative results are presented. RT-PCR was undertaken on RNA prepared from *S. aureus* strain RN6390 grown aerobically in TSB to an OD_{600} of 2, as described previously[16] using primer pairs listed in Table S3, and was performed twice (on the same biological sample – results presented in Fig S14b are representative).

Strain and plasmid construction: All oligonucleotide primers used in this study and cloning strategies to generate the strains and plasmids are outlined in Table S3. In-frame deletions of *S. aureus* genes were performed by allelic exchange using pIMAY[46]. For each gene, the upstream and downstream regions including at least the first three and last three codons were amplified from RN6390 genomic DNA using primers listed in Table S3. Clones were selected in *E. coli*, verified by DNA sequencing and introduced into *S. aureus* RN6390 strains by electroporation. Chromosomal deletions were verified by amplification of the genomic region from isolated genomic DNA (GeneElute Bacterial Genomic DNA Kit, Sigma Aldrich) and DNA sequencing of the amplified products.

To construct strains specifying chromosomally-encoded erythromycin resistance, the erythromycin resistance gene, *ermC*, was integrated into the RN6390 genome after base pair 14208 as this region was found to be devoid of transcriptional activity in the closely related strain NCTC 8325-4[51]. A synthetic construct comprising *ermC* from *Staphylococcus lentus* plasmid pSTE2 under control of the *rpsF* promoter from *Bacillus subtilis* (purchased from Biomatik; sequence given in Fig S16) was cloned into pIMAY and integrated into the chromosome giving RN6390::*ermC*. Integration of the resistance gene was confirmed by sequencing and by testing for growth in the presence of 5 µg/ml erythromycin. Subsequently the *ermC* cassette was transduced from RN6390::*ermC* into other strains using phage ϕ11 as described[52]. Transduction was confirmed by PCR amplification using oligonucleotides Intctrl1 and Intctrl2 (Table S3).

Bacterial competition experiments: Overnight cultures of the indicated strains were subcultured in TSB (supplemented with 2µM hemin for attacker strains) and antibiotics as required and cultured with shaking at 37°C. Once OD_{600} of 0.5 was reached, induction of EsaD-HA and EsaG-His production in the attacker strains was initiated by the addition of 500 ng/ml of ATC. When cells reached OD_{600} of 2, 20ml of attacker strain, and 1ml of prey were separately harvested and resuspended in 1ml of TSB. 100ul of resuspended attacker cells were mixed with the same volume of prey cells (giving a 20:1 ratio) and incubated at 37°C with shaking for 16 h in sterile Eppendorf tubes. Co-cultures were then serially diluted in TSB and plated on selective agar (LB + 5 µg/ml erythromycin as all prey strains carried chromosomally-integrated *ermC* conferring resistance to erythromycin) for colony-forming unit determination. For experiments where plasmid-encoded EsaG-His was produced in prey cells, once strain RN6390 pEsaG-His reached an OD_{600} of 0.5, EsaG-His production was initiated by the addition of 250 ng/ml of ATC and cultured until an OD_{600} of 2 was reached after which they were used as prey as described above.

TUNEL assay for DNA fragmentation: DNA fragmentation was detected in fixed cells of *E. coli* BL21(DE3) harbouring pT7.5esaD and pT7.5esaDG using the Deadend fluorometric TUNEL system kit (Promega). This was undertaken on two biological replicates and

representative results are shown in FigS3 c and d. Following induction of EsaD production by treating cell cultures with 1mM IPTG for 3 hours, cells were pelleted, washed twice with PBS and fixed with 4% formaldehyde in PBS for 30 minutes on ice. Following a further wash with PBS, cells were permeabilized with 1.5% triton X-100 solution in PBS for 1 hour on ice and stored in 70% ice cold ethanol at -20 ^{0}C overnight. The following day cells were spun down, washed and resuspended in equilibration buffer for 1 hour at 37^{0}C. The cells were incubated in the dark for 2 hours with fluorescein 12-dUTP and recombinant terminal deoxynucleotidyl transferase (rTdT), after which the reaction was quenched by addition of 2x SCC buffer, and the cells washed with PBS. Following this fluorescein-labelled cells were spotted onto poly-D-lysine-treated slides and analysed by fluorescence microscopy using a Zeiss fluorescence microscope with a 100x oil objective and images captured using an AXIO camera (Zeiss), or detected and quantitated directly by flow cytometry (Flow cytometry Facility, Dundee University). Negative control samples were treated identically except that no rTdT was added.

Purification of the EsaD nuclease domain: *E. coli* strain M15[prep4] harbouring pQE70-EsaG-EsaG-EsaD$_{421\text{-}614}$-His or pQE70-EsaG-EsaG-EsaD$_{421\text{-}614}$(H528A)-His was cultured to OD_{600} of 0.5 at 37ºC, after which 1mM IPTG was added to each culture. An aliquot of the cells were harvested after 4 hours induction and resuspended in lysis buffer and the remainder of the cells were pelleted and frozen at -80°C for 30 mins. The nuclease domain of EsaD was subsequently purified following a protocol based on that described at http://openwetware.org/wiki/Knight:Purification_of_His-tagged_proteins/Denaturing_with_refolding with some modifications, and was undertaken once for each variant (Fig 2e). Briefly, the thawed pellets were resupended in lysis buffer (8 M Urea, 100 mM NaH_2PO_4, 10mM Tris·HCl, 10mM imidazole, 1 mM PMSF, pH 8.0) and sonicated for 2 mins on ice. The cell lysate was centrifuged at 13,200 × *g* for 30 min at 4 °C. Nickel affinity resin (Biorad) was equilibrated with lysis buffer and added to the cleared lysate and gently mixed on a rotary shaker for 2 h at 4 °C. The resin was washed five times with denaturing wash buffer (8 M Urea, 100 mM NaH_2PO_4, 150mM NaCl, 20mM imidazole, 1 mM PMSF, pH 8.0) followed by five washes with native wash buffer (50 mM NaH_2PO_4, 500mM

NaCl, 20mM imidazole, 1 mM PMSF, pH 8.0). Finally, the bound protein was eluted into 200 µl of elution buffer (50 mM NaH_2PO_4, 500mM NaCl, 250mM imidazole, 1 mM PMSF, pH 8.0). For nuclease assays, 800 ng of plasmid pT18 was incubated with 0.4 µg of purified $EsaD_{421-614}$-His, purified $EsaD_{421-614}$(H528A)-His or an equivalent volume of elution buffer at 37°C for 1 hour in a final volume of 20µl the presence of either 50mM $MgCl_2$ or $ZnCl_2$ as indicated, after which the DNA was analysed by agarose gel electrophoresis. These assays were each performed three times and representative results are presented in Figs 2f and S5.

Cell fractionation, crosslinking and western blotting: For the isolation of cell and supernatant fractions to assess secretion activity, *S. aureus* strains were subcultured at 1/100 from an overnight grown pre-culture into fresh TSB medium. At OD_{600nm} of 2, cells were harvested and the supernatant samples precipitated with trichloroacetic acid in the presence of deoxycholate, as described previously[16]. Harvested cell samples were washed once with PBS buffer, normalized to an OD_{600} of 2 in PBS and lysed by addition of 50 µg/ml lysostaphin with incubation at 37°C for 30 min. All samples were mixed with an equal volume of LDS buffer and boiled for 10 min prior to analysis. For the secretion experiments shown in Fig 1b, 1c, 1e, S9a, S9b and S14 representative images are shown from at least two biological replicates, and in Figs 1d, 3d, 3e, S7 and S8 representative images are shown from at least six biological replicates. The fractionation of cells to give cell wall, membrane and cytoplasmic fractions was undertaken as described by[16]. Carbonate-washing of membranes was undertaken according to[53]. All fractionation experiments (Figs 1f, 3a and S6) were undertaken at least twice on separate biological samples, with representative results presented.

Formaldehyde crosslinking of cells was undertaken as described previously[17] and crosslinking experiments were performed twice on separate biological samples – the results presented in Fig 3b and c are representative results. Western blotting was performed according to standard protocols using the following antibody dilutions: α-EsxA[16] 1:2500, α-EsxC[16] 1:2000, α-EssC[16] 1:10000, α-TrxA[54] 1:25000, α-SrtA (Abcam, catalogue number ab13959) 1:3000, α-HA (HRP-conjugate, Sigma catalogue number H6533) 1:10000, α-His (HRP-conjugate, Abcam

catalogue number ab184607) 1:10000, α-Myc (HRP-conjugate, Invitrogen catalogue number R951-25) 1:5000, and goat anti Rabbit IgG HRP conjugate (Bio-Rad, catalogue number 170-6515) 1:10000.

Protein purification by nickel affinity isolation or immunoprecipitation: Cells of *E. coli* or *S. aureus*, grown as described in the figure legends, were harvested and resuspended in either ice cold (i) resuspension buffer (200 mM NaCl, 20 mM HEPES, pH 7.2) for Ni affinity purification or (ii) phosphate-buffered saline (PBS) for immunoprecipitation. Samples were then supplemented with a few flakes of DNase I, 1 mM PMSF and either lysozyme (for *E. coli*) or lysostaphin (for *S. aureus*). The samples were incubated at 37°C for 0.5-1 hour with gentle mixing on a rotating wheel after which cells were lysed by French press (for *E. coli*) or sonication (*S. aureus*). Unbroken cells and cellular debris was pelleted by centrifugation at 17,000 *g* and 4°C for 30 min and the supernatant was retained as the cell lysate. All protein purification experiments shown in Figs 2c, 2d, 3b, 3f, 3g, S4, S10b, S11 and S12 were performed at least twice with different biological replicates.

For Ni-affinity purification, 100 µl Ni-NTA resin (Biorad, catalogue number 156-0131) was equilibrated by washing twice in 1 ml ice cold wash buffer (200 mM NaCl, 15 mM imidazole, 20 mM HEPES, pH 7.2). Cell lysate was diluted to 5 µg/µl protein in ice cold wash buffer (200 mM NaCl, 15 mM imidazole, 20 mM HEPES, pH 7.2) in a final volume of 1.2 ml, added to the equilibrated Ni-NTA resin and gently agitated for 1 hour at 4°C. The Ni-NTA resin was pelleted by centrifugation, washed four times with 1 ml ice cold wash buffer and finally resuspended in 100 µl elution buffer (200 mM NaCl, 300 mM imidazole, 20 mM HEPES, pH 7.2), mixing at 4°C for 1 hour. The Ni-NTA resin was pelleted, the supernatant carefully removed and retained as the eluted fraction.

For immunoprecipitation, 40-100 µl of Anti-HA Agarose bead suspension (Sigma, catalogue number A2095) were pelleted, washed twice with PBS and mixed with cell lysate which was diluted to 5 µg/µl in 200 µl PBS. The suspension was incubated with agitation for at least 1 hour at 4°C after which the beads were pelleted and washed four times with 1 ml ice cold wash

PBS. After the final wash the supernatant was aspirated to leave ~30 µl of PBS above the beads for final resuspension.

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609 **Main text figure legends**

610 **Fig 1. EsaD is a substrate of the T7SS.** a. The *ess* locus – genes coding for core components
611 of the secretion machinery are in green, secreted components yellow and proteins
612 investigated as part of this study in white. b. and c. EsaD is not required for secretion of EsxA
613 and EsxC – (b) the RN6390 wild-type or isogenic deletion strains, as indicated, were cultured
614 in TSB medium to OD_{600} of 2 or (c) the indicated strains harbouring pRAB11 (empty) or
615 pRAB11-EsxC were cultured in TSB medium to OD_{600} of 0.5, then supplemented with ATC
616 (50ng/ml; to induce plasmid-encoded gene expression) until OD_{600} of 2. Cells were pelleted
617 and the supernatant (sn) was retained as the secreted protein fraction. Samples of the
618 supernatant and whole cells (an equivalent of 200μl of culture supernatant and 10μl of cells
619 adjusted to OD1) were separated on 12 % bis-Tris gels and immunoblotted with the indicated
620 antisera (with TrxA serving as a cytoplasmic control). Note that the samples were run on the
621 same gel but intervening lanes have been spliced out (unspliced version is shown in Fig S9).
622 (d) and (e). EsaD is secreted in an *essC*-dependent manner. The indicated *S. aureus* strains
623 harbouring pRAB11 (empty) or pRAB11-EsaD(H528A)-HA were treated as described in (c)
624 except that 250ng/ml ATC was used to induce EsaD(H528A)-HA production and an equivalent
625 of 250μl of supernatant and 10μl of cells adjusted to OD_{600} of 1 were loaded f. Cells of the wild
626 type *S. aureus* strain, RN6390, harbouring pRAB11 or pRAB11-EsaD(H528A)-HA from (d)
627 were fractionated into cytoplasmic (cyt) and membrane (m) fractions. Samples of each fraction
628 (20μl aliquot of cyt, and 2mg of membrane) were separated on 12% bis-Tris gels and
629 immunoblotted using either anti-HA, anti-EssB (membrane protein control) or anti-TrxA
630 (cytoplasmic control) antisera.
631

632 **Fig 2. EsaDG form a nuclease toxin-antitoxin pair.** a. EsaD is toxic to *E. coli*. *E. coli*
633 BL21(DE3) harbouring pT7.5 (empty vector), pT7.5-esaD(V584Y) or pT7.5-esaDG was
634 cultured to OD_{600} 0.5, supplemented with 1 mM IPTG (time zero) and OD_{600} measured at 1 hr
635 intervals (*n*=3 biological replicates, error bars are $\pm$ SD). b. – d. EsaG interacts with the

636 nuclease domain of EsaD. b. Interactions between pT25-EsaG and EsaD variants fused to
637 pT18 assessed by β-galactosidase activity assay in *E. coli* BTH101. BTH101 harbouring pT25
638 and pT18 was the negative control. Error bars are ± SD (*n*=3 biological replicates). Student's
639 *t*-test gives *p* values < 0.00001 for EsaD/EsaG and $EsaD_{421-614}$/EsaG relative to the negative
640 control. Inset shows the same strain/plasmid combinations on MacConkey maltose plates. c.
641 and d. Top two panels: *S. aureus* RN6390 carrying c. pRAB11 (empty vector), pRAB11-EsaG-
642 HA or pRAB11-EsaD(H528A)-His-EsaG-HA, or e. pRAB11 (empty), pRAB11-EsaG-HA,
643 pRAB11-$EsaD_{421-614}$(H528A)-His or pRAB11-$EsaD_{421-614}$(H528A)-His-EsaG-HA was cultured
644 to OD_{600} of 0.5, then supplemented with ATC (500ng/ml). Cells were harvested at OD_{600} 3,
645 lysed and histidine-tagged EsaD purified. Cell lysate (load) and eluted fractions (20μl of each)
646 were analysed by western blot with anti-His and anti-HA antisera. Bottom two panels show
647 repeat experiments of: c. the EsaD(H528A)-His-EsaG-HA co-purification or d. the $EsaD_{421-}$
648 $_{614}$(H528A)-His-EsaG-HA co-purification. Samples of load (10μl), flow through (20μl), final
649 wash (30μl) and elution fraction (30μl) were analysed using the same antisera. Coomassie-
650 stained samples of the load and elute fractions are shown in Fig S4. e. *E. coli* M15[prep4]
651 harbouring pQE70 alone (empty) pQE70-EsaG-EsaG-$EsaD_{421-614}$-His or pQE70-EsaG-EsaG-
652 $EsaD_{421-614}$(H528A)-His were cultured to OD_{600} of 0.5 and supplemented with 1mM IPTG. An
653 aliquot was harvested after 4 hours' induction and resuspended in lysis buffer. His-tagged
654 EsaD nuclease domain (wild type or H528A variant) was purified in the presence of 8M urea,
655 refolded and eluted as described in Methods. 10μl of each sample were separated (12% bis-
656 Tris gel) and stained using coomassie instant blue. f. EsaD is a Mg^{2+}-dependent DNase.
657 Plasmid DNA was incubated with purified $EsaD_{421-614}$-His, $EsaD_{421-614}$(H528A)-His or buffer
658 alone with either 50mM $MgCl_2$ or $ZnCl_2$, after which the DNA was analysed by agarose gel
659 electrophoresis.

660

661

Fig 3. EsaE is co-secreted with EsaD and together with EsaG they form a ternary complex. a – c. EsaE interacts with EsaD. Interactions between a. pT25-EsaD(H528A) and the indicated fusions to pT18 or c. pT25-EsaE and $EsaD_{1-420}$ or $EsaD_{421-614}$(H528A) fused to pT18 as well as pT18-EsaE and full length EsaD(H528A) fused to pT25 assessed by β-galactosidase activity assay in BTH101. BTH101 harbouring pT25 and pT18 was the negative control. Error bars are ± SD (*n*=3 biological replicates). Student's *t*-test gives *p* values < 0.00001 for EsaD(H528A)/EsaE, EsaD(H528A)/EsaG, EsaD/EsaE, and $EsaD_{1-420}$/EsaE relative to the negative control. Inset shows the same strain and plasmid combinations on MacConkey maltose plates. b. Top two panels - *S. aureus* RN6390 carrying pRAB11 (empty), pRAB11-EsaE-HA or pRAB11-EsaD(H528A)-His-EsaE-HA was cultured to OD_{600} of 0.5 supplemented with 500ng/ml ATC and harvested at OD_{600} of 3. Cells were lysed and histidine-tagged EsaD(H528A) was purified. Cell lysate (load) and eluted fractions (20μl of each) were analysed by western blot with anti-His and anti-HA antisera. Coomassie-stained samples of these fractions are shown in Fig S4. Bottom two panels show repeats of EsaD(H528A)-His-EsaE-HA co-purification. Samples of load (10μl), flow through (20μl), final wash (30μl) and elution fraction (30μl) were analysed using the same antisera. d and e. EsaE is co-secreted with EsaD. *S. aureus* RN6390 harbouring pRAB11 (empty) and either d. pRAB11-EsaE-His or e. pRAB11-EsaD-His-EsaE-HA was cultured to OD_{600} of 0.5 supplemented with 250ng/ml ATC and harvested at OD_{600} of 3. Samples of supernatant and cells (equivalent to 250μl supernatant and 10μl cells adjusted to OD_{600} of 1) were separated on 12% bis-Tris gels and immunoblotted with the indicated antisera. f. EsaE, EsaD and EsaG form a ternary complex. *E. coli* M15[pRep4] carrying pQE70 (empty) or pQE70- EsaE-HA-EsaD(H528A)-Myc-EsaG-His was cultured to OD_{600} of 0.5, supplemented with 2 mM IPTG for 4 hours, harvested and lysed. HA-tagged EsaE was purified and 10μl, 25μl and 35μl of the elution fractions were analysed by western blot with anti-HA, anti-His and anti-myc antibodies, respectively. Coomassie-stained samples of these fractions are shown in Fig S10. g. Samples of load (10μl), flow through (20μl), final wash (30μl) and elution fraction (30μl) from the EsaE-HA-

689 EsaD(H528A)-Myc-EsaG-His co-purification experiment shown in f. were analysed by western
690 blotting with the same antisera.

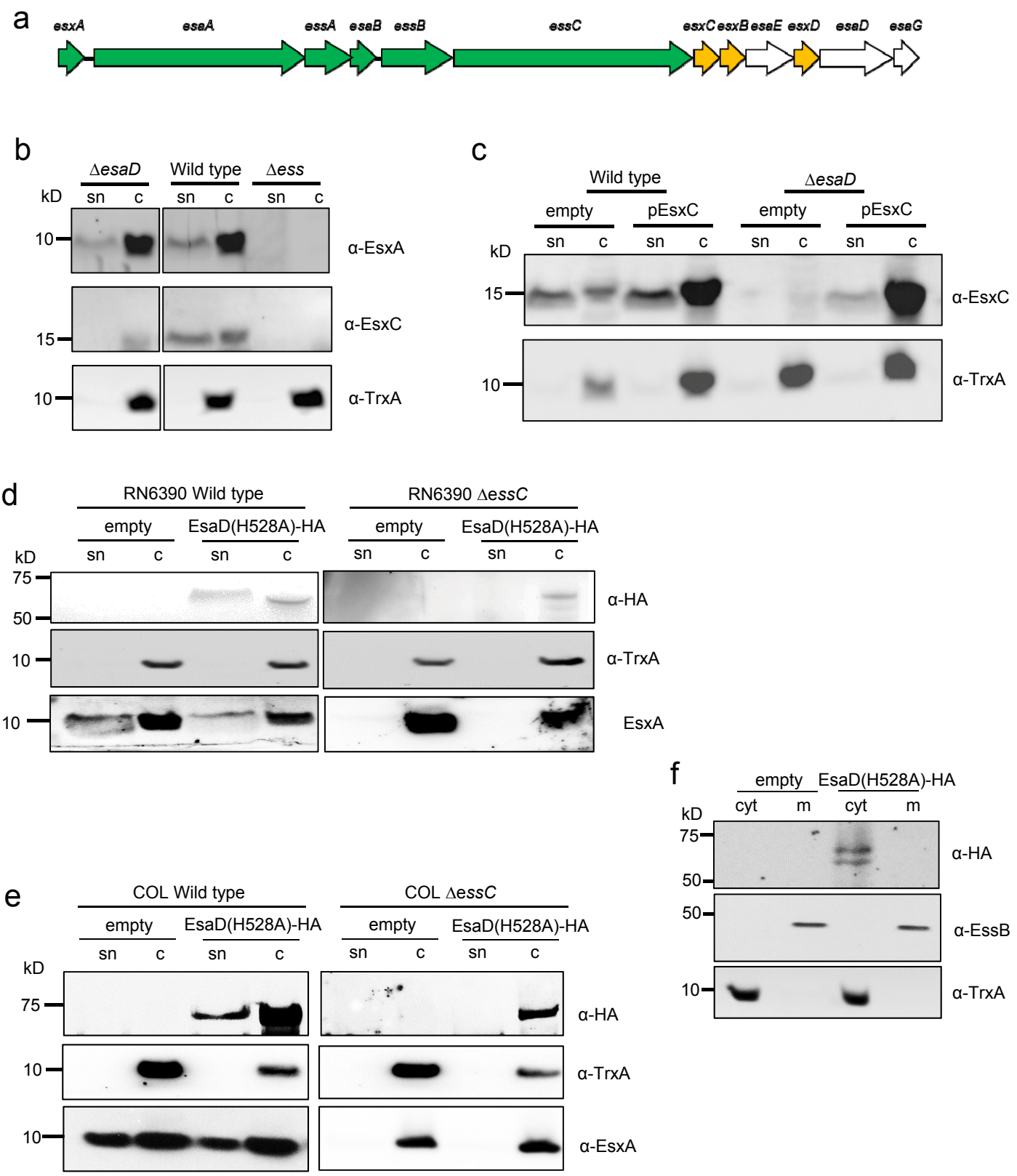
691

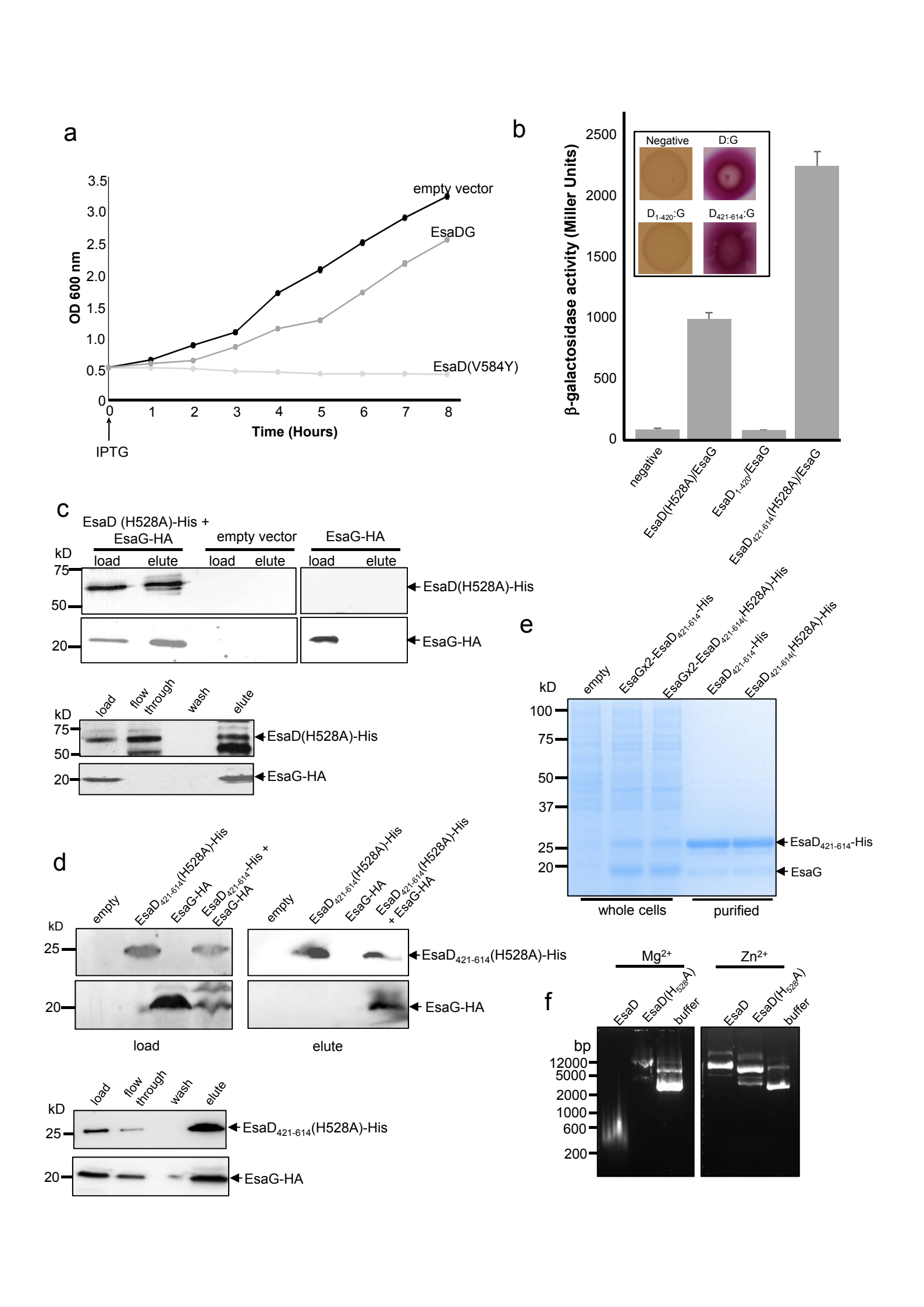
692 **Fig 4. EsaE is a membrane-associated protein that interacts with multimeric EssC.** a. A
693 proportion of EsaE is bound to the membrane. The *S. aureus* wild type strain, RN6390,
694 harbouring pRAB11 (empty) or pRAB11-EsaE-His was cultured in TSB medium to OD_{600} of
695 0.5, supplemented with ATC (250ng/ml) and harvested at OD_{600} of 2. Cells were fractionated
696 into cell wall (cw), cytoplasmic (cyt) and membrane (m) fractions. An aliquot of the membrane
697 fraction was washed with 0.2 M Na_2CO_3 (m+). Samples of each fraction (20µl aliquot of cw and
698 cyt; 2mg of membrane) were separated on 12% bis-Tris gels and immunoblotted using either
699 anti-His or anti-sortase A (SrtA) antisera. b and c. EsaE crosslinks to a multimeric form of
700 EssC. b. Whole cells of the *S. aureus* wild type (RN6390), or c. the wild type and the isogenic
701 *essC* deletion strain, as indicated, harbouring pRAB11 (empty) or pRAB11-EsaE-His were
702 cultured in TSB medium to OD_{600} of 0.5 supplemented with ATC (250ng/ml) and at OD_{600} of
703 2, cells were incubated with paraformaldehyde (PFA) as described under Methods. Following
704 quenching, cells were lysed and membrane fractions prepared, and membrane protein (1mg
705 for samples from the wild type strain, 10mg for samples from the *essC* strain) loaded on b. a
706 bis-Tris gel containing 12% acrylamide or c. SDS-gels containing 5 % acrylamide (bottom
707 panel in part C showing EsaE-His monomer is 12% bis-Tris gel) and analysed by western blot
708 with the indicated antisera. d. Model for EsaD synthesis and secretion. Following synthesis of
709 EsaD (shown in green), ***1*** EsaE binds to the N terminal region of the protein and ***2*** EsaG binds
710 to the nuclease domain to prevent activity against the DNA of the producing cell. ***3*** the ternary
711 complex is targeted to the secretion machinery facilitated by the interaction of EsaE with
712 multimeric EssC. ***4*** EsaG is released from EsaD during the transport step and ***5*** the EsaD-
713 EsaE complex is secreted out of the cell via the T7SS.

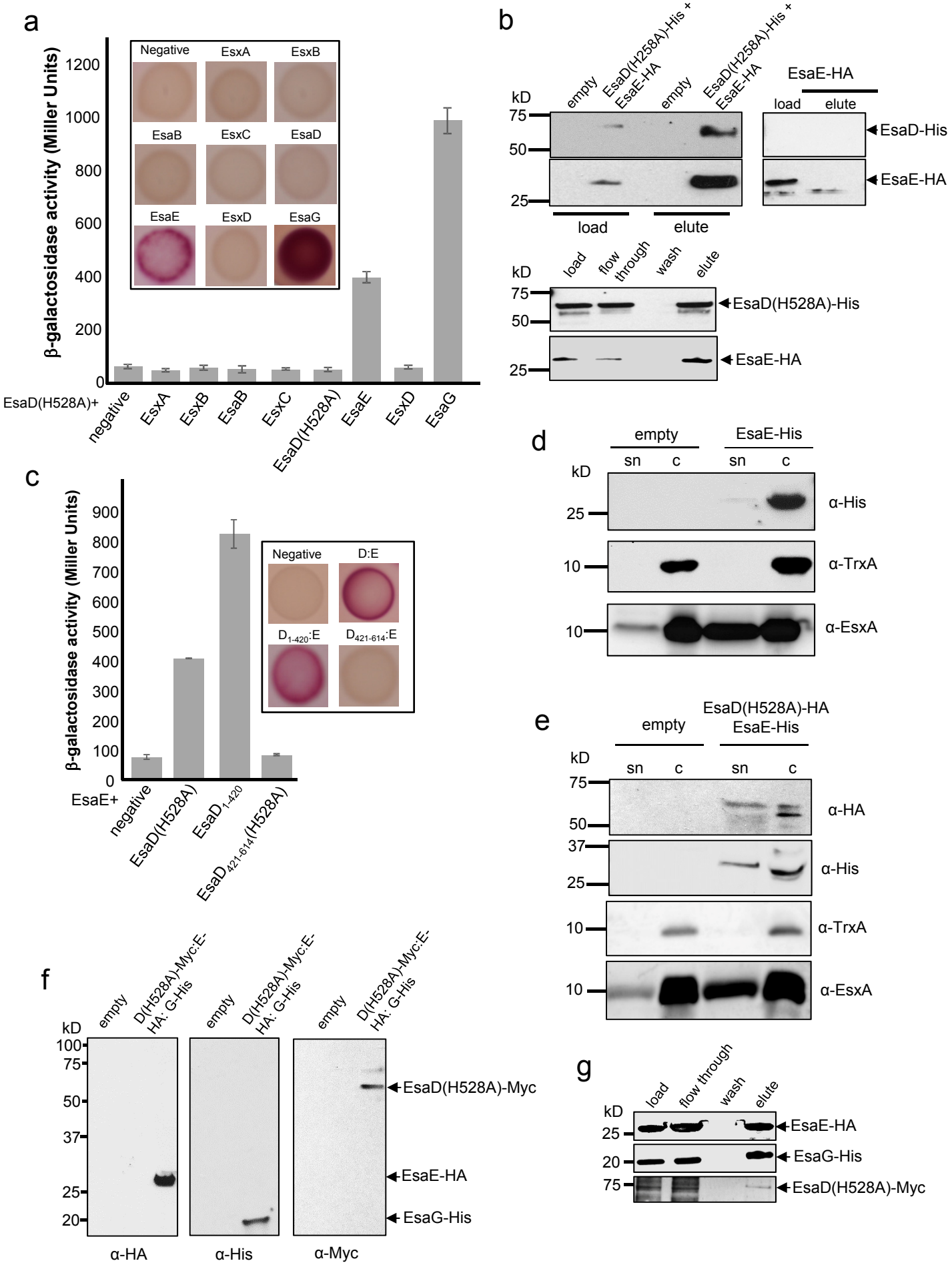
714

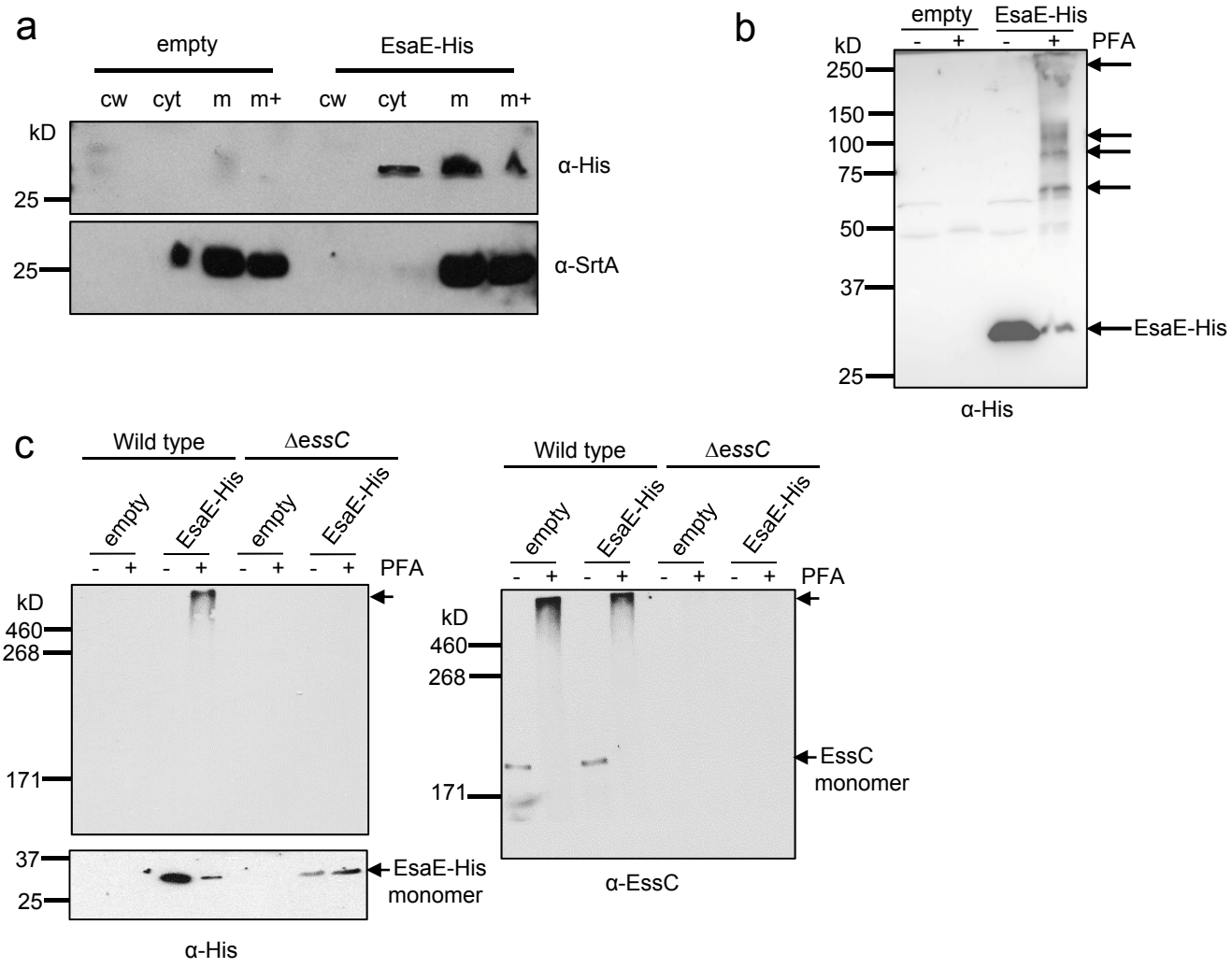
715

716 **Fig 5. Secreted EsaD kills sensitive strains of *S. aureus*.** a. EsaG homologues are encoded
717 in *S. aureus* strains that lack *esaD*. DUF600-family proteins encoded at the *ess* loci in *S.*
718 *aureus* strains NCTC8325 (parental strain of RN6390), MRSA252, ST398 and EMRSA15.
719 Genes encoding DUF600 proteins are shaded in purple, *essC* is shaded green and *esaD* grey.
720 The two genes shaded in brown are highly conserved across all strains and define the 3'
721 boundary of the *ess* locus[18]. b. and c. Interactions between pT25-EsaD(H528A) and DUF600
722 proteins; b. from strains ST398, MRSA252 and EMRSA15, and c. from strain NCTC8325. In
723 each case the DUF600 reading frame was fused to pT18 and interaction with full length EsaD
724 fused to pT25 assessed by β-galactosidase activity assay in *E. coli* BTH101. BTH101
725 harbouring pT25 and pT18 was the negative control. Error bars are ± SD (*n*=3 biological
726 replicates). Student's *t*-test gives *p* values < 0.00001 relative to the negative control. Insets
727 shows the same strain and plasmid combinations on MacConkey maltose plates. d. *In vitro*
728 growth competition assays between the indicated attacker and prey strains in liquid medium.
729 In each case the attacker strain (COL or COLΔ*ess*) overproduced EsaD-HA or EsaD(H528A)-
730 HA along with EsaG-His as described in Methods, and was incubated with either RN6390,
731 RN6390Δ*00268-00278* or RN6390 pEsaG-His as prey, as indicated. To the right, the three
732 prey strains incubated with COL pEsaD-HA-EsaG-His as attacker are replotted next to each
733 other to allow a more direct comparison. In all experiments five biological replicates of each
734 attacking strain was used against a single culture of prey. Bars represent the average value
735 of c.f.u. of prey bacteria at the end of the experiment. Asterisks indicate significant differences
736 in c.f.u. * *p* value < 0.05; ** *p* value < 0.005, *** *p* value < 0.0005. Comparison of RN6390 with
737 RN6390Δ*00268-00278* survival when COL pEsaD-HA-EsaG-His was used as attacker was
738 not significant (*p* = 0.069). Error bars are ± SD (*n* = 5 biological replicates).


a
esxA
esaA
essA
esaB
essB
essC
esxC
esxB
esaE
esxD
esaD
esaG
b
ΔesaD
Wild type
Δess
kD
sn
c
10
α-EsxA
15
α-EsxC
10
α-TrxA
c
Wild type
ΔesaD
empty
pEsxC
kD
15
α-EsxC
10
α-TrxA
d
RN6390 Wild type
RN6390 ΔessC
empty
EsaD(H528A)-HA
kD
75
50
10
α-HA
α-TrxA
EsxA
f
empty
EsaD(H528A)-HA
cyt
m
kD
75
50
α-HA
α-EssB
10
α-TrxA
e
COL Wild type
COL ΔessC
empty
EsaD(H528A)-HA
kD
75
10
α-HA
α-TrxA
α-EsxA


a
OD 600 nm
Time (Hours)
empty vector
EsaDG
EsaD(V584Y)
IPTG
b
β-galactosidase activity (Miller Units)
Negative
D:G
D1-420:G
D421-614:G
negative
EsaD(H528A)/EsaG
EsaD1-420/EsaG
EsaD421-614(H528A)/EsaG
c
EsaD (H528A)-His + EsaG-HA
empty vector
EsaG-HA
load
elute
kD
EsaD(H528A)-His
EsaG-HA
flow through
wash
d
empty
EsaD421-614(H528A)-His
EsaG-HA
EsaD421-614-His + EsaG-HA
EsaD421-614(H528A)-His + EsaG-HA
load
elute
e
empty
EsaGx2-EsaD421-614-His
EsaGx2-EsaD421-614(H528A)-His
EsaD421-614-His
EsaD421-614(H528A)-His
EsaD421-614-His
EsaG
whole cells
purified
f
Mg2+
Zn2+
EsaD
EsaD(H528A)
buffer
bp


a
β-galactosidase activity (Miller Units)
1200
1000
800
600
400
200
0
Negative
EsxA
EsxB
EsaB
EsxC
EsaD
EsaE
EsxD
EsaG
EsaD(H528A)+
negative
EsxA
EsxB
EsaB
EsxC
EsaD(H528A)
EsaE
EsxD
EsaG
b
kD
75
50
25
empty
EsaD(H258A)-His +
EsaE-HA
empty
EsaD(H258A)-His +
EsaE-HA
load
elute
EsaE-HA
load
elute
EsaD-His
EsaE-HA
kD
75
50
25
load
flow through
wash
elute
EsaD(H528A)-His
EsaE-HA
c
β-galactosidase activity (Miller Units)
900
800
700
600
500
400
300
200
100
0
Negative
D:E
D1-420:E
D421-614:E
EsaE+
negative
EsaD(H528A)
EsaD1-420
EsaD421-614(H528A)
d
empty
EsaE-His
sn
c
sn
c
kD
25
10
10
α-His
α-TrxA
α-EsxA
e
empty
EsaD(H528A)-HA
EsaE-His
kD
sn
c
sn
c
75
50
37
25
10
10
α-HA
α-His
α-TrxA
α-EsxA
f
kD
100
75
50
37
25
20
empty
D(H528A)-Myc:E-HA: G-His
empty
D(H528A)-Myc:E-HA: G-His
empty
D(H528A)-Myc:E-HA: G-His
EsaD(H528A)-Myc
EsaE-HA
EsaG-His
α-HA
α-His
α-Myc
g
load
flow through
wash
elute
kD
25
20
75
EsaE-HA
EsaG-His
EsaD(H528A)-Myc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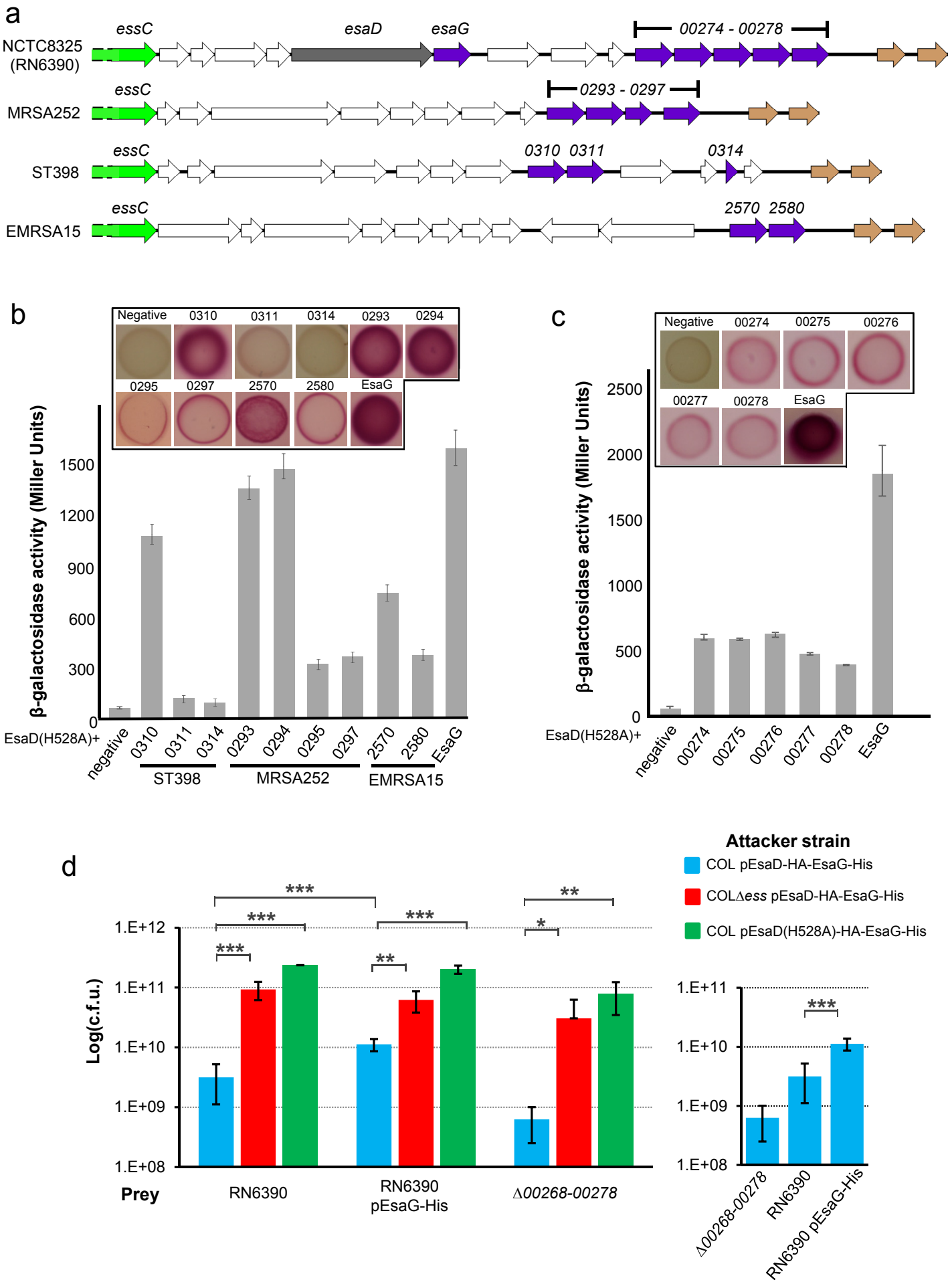
EsaE-His
cw cyt m m+ cw cyt m m+
kD
25
α-His
25
α-SrtA
b
empty
EsaE-His
- + - +
PFA
kD
250
150
100
75
50
37
25
EsaE-His
α-His
c
Wild type
ΔessC
empty
EsaE-His
empty
EsaE-His
- + - + - + - +
PFA
kD
460
268
171
37
25
EsaE-His
monomer
α-His
Wild type
ΔessC
empty
EsaE-His
empty
EsaE-His
- + - + - + - +
PFA
kD
460
268
171
EssC
monomer
α-EssC
d
EsaE
LxG
nuclease
5
cell exterior
cytoplasm
EssC
4
3
EsaG
EsaG
EsaE
EsaE
EsaG
LxG
nuclease
1
LxG
nuclease
2
LxG
nuclease


a
NCTC8325 (RN6390)
essC
esaD
esaG
00274 - 00278
MRSA252
0293 - 0297
ST398
0310 0311
0314
EMRSA15
2570 2580
b
Negative
0310
0311
0314
0293
0294
0295
0297
2570
2580
EsaG
β-galactosidase activity (Miller Units)
EsaD(H528A)+
negative
ST398
MRSA252
EMRSA15
c
Negative
00274
00275
00276
00277
00278
EsaG
d
Attacker strain
COL pEsaD-HA-EsaG-His
COLΔess pEsaD-HA-EsaG-His
COL pEsaD(H528A)-HA-EsaG-His
Log(c.f.u.)
Prey
RN6390
RN6390 pEsaG-His
Δ00268-00278

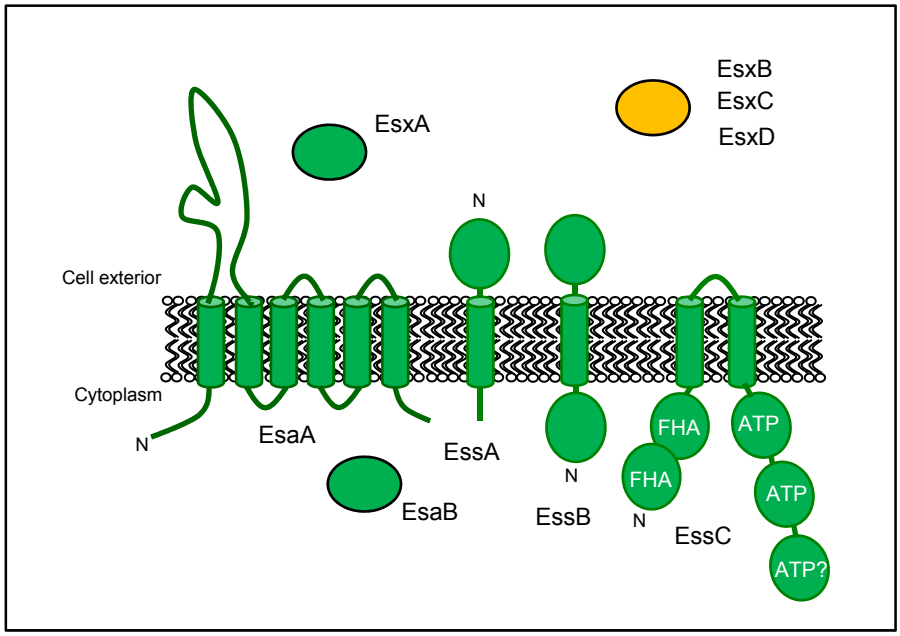
The Ess/Type VII secretion system of *Staphylococcus aureus* secretes a nuclease toxin that targets competitor bacteria

Supplementary information

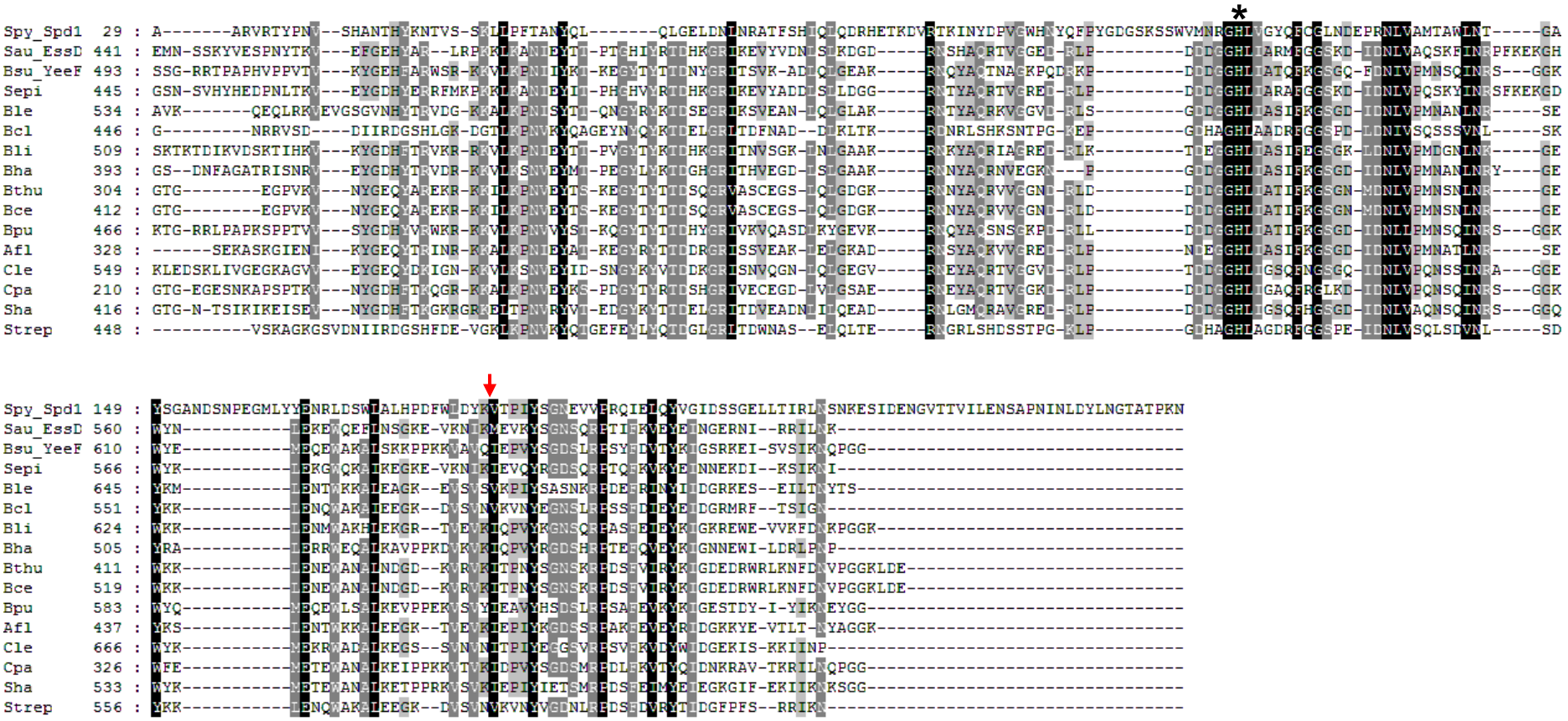
Supplementary Figures 1-16

Supplementary Tables 1-3

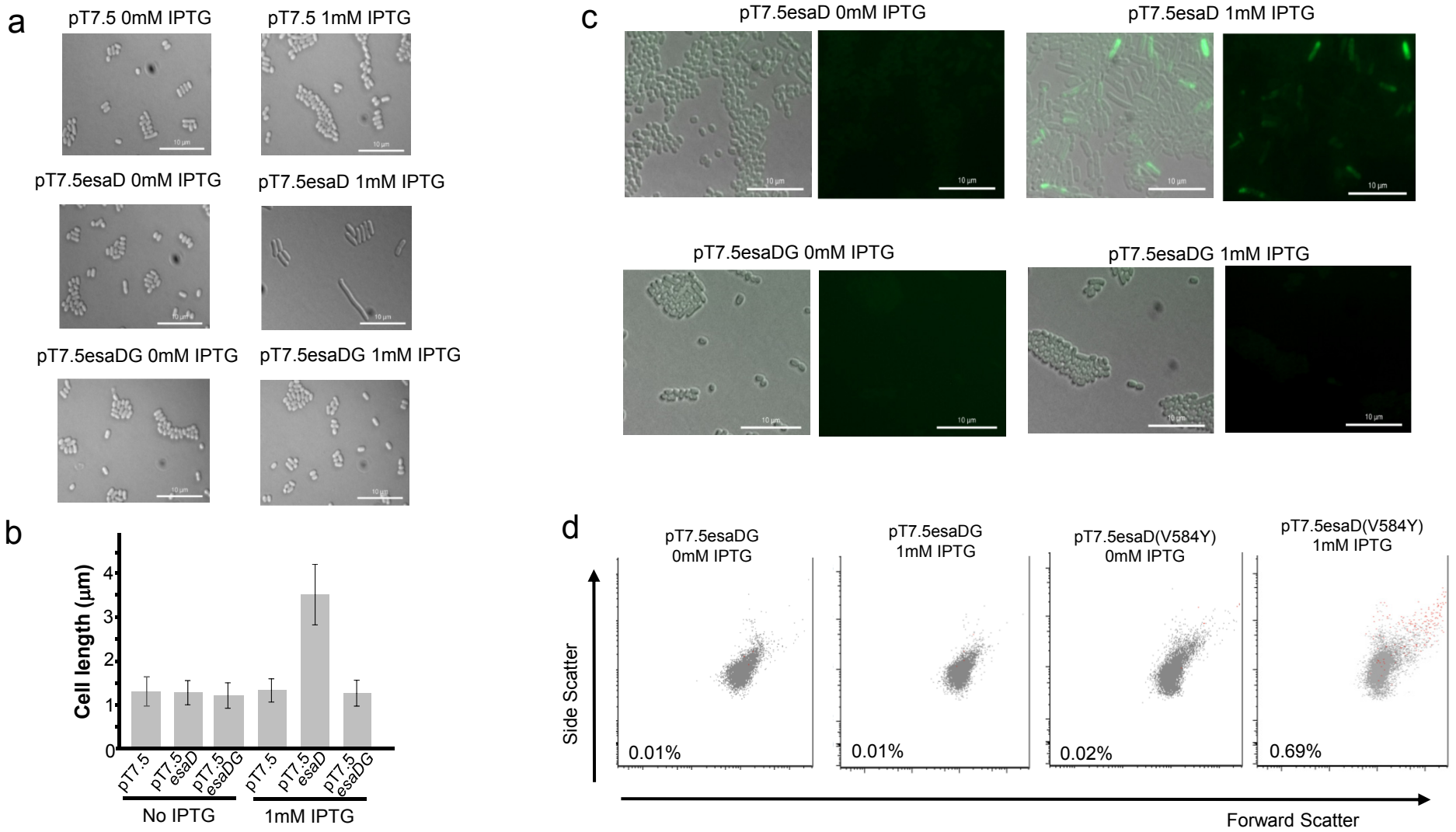
Supplementary References



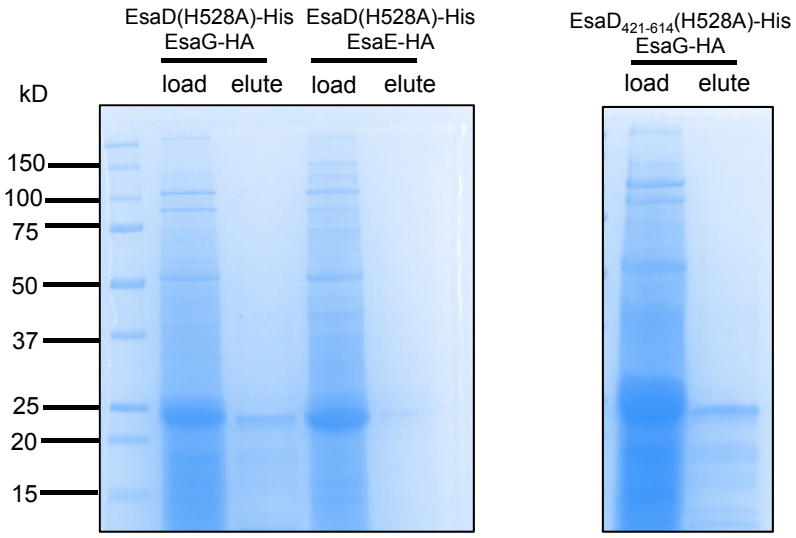
Supplementary Figure 1. Localization and predicted topologies of Ess components in *S. aureus* strain RN6390. Essential components of the secretion machinery are shown in green and known secreted substrates in yellow. FHA – forkhead associated domain. ATP – P-loop ATP-binding domain.



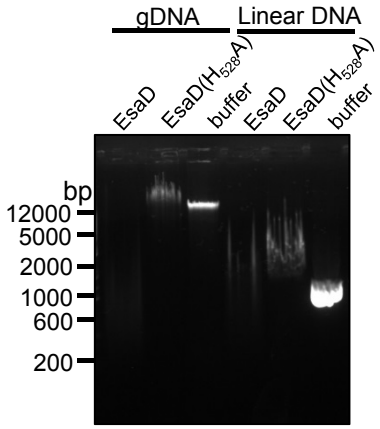
Supplementary Figure 2. EsaD has a predicted nuclease domain at its C-terminus. Alignment of *S. aureus* EsaD with Orthologs identified using BlastP [10]. Multiple sequence alignment was generated using Clustal Omega [11] with default settings. The *Streptococcus pyogenes* Spd1 protein, for which nuclease activity has been described [12], was included as a reference for the Endonuclease_NS_2 family (PFAM 13930). The position of the catalytically active Histidine 121 in Spd1 (H528 for EsaD) is marked with an asterisk. The position of EsaD V584 that was substituted to a tyrosine in this work is indicated by a red arrow. Abbreviations: Afl, *Anoxybacillus flavithermus*; Bce, *Bacillus cereus*; Bcl, *B. clausii*; Bha, *B. halodurans*; Ble, *B. lentocellum*; Bli, *B. licheniformis*; Bpu, *B. pumilus*; Bsu, *B. subtilis*; Cle, *Clostridium lentocellum*; Cpa, *Cohnella panacarvi*; Sau, *Staphylococcus aureus*; Sepi, *S. epidermidis*; Sha, *Sediminibacillus halophilus*; Spy, *Streptococcus pyogenes*; Strep, *Streptococcus sp*.



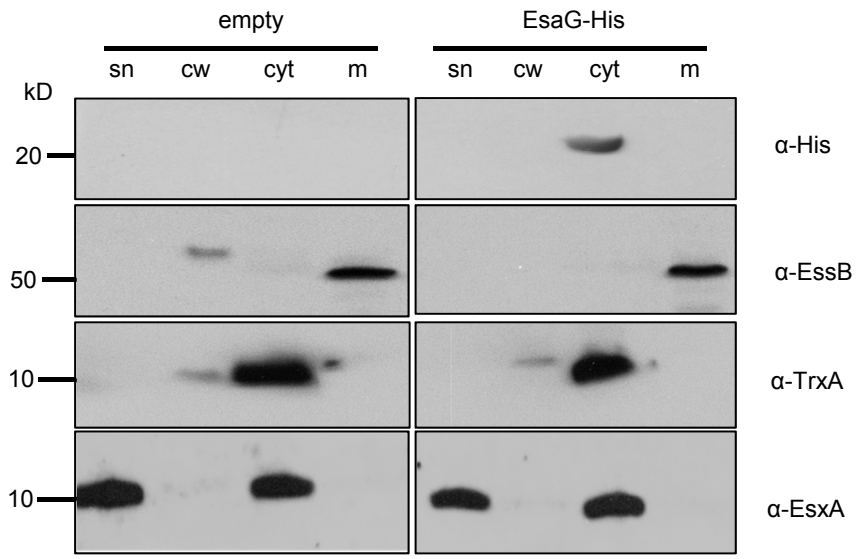
Supplementary Figure 3. Microscopy analysis of *E. coli* cells producing EsaD. *E. coli* strain BL21(DE3) harbouring plasmids pT7.5, pT7.5esaD(V584Y) or pT7.5esaDG was cultured in LB at 37^{0}C to OD_{600} 0.5, an aliquot of cells was removed and the remainder of the sample was supplemented with 1 mM IPTG for a further three hours. a. Cells were analysed by light microscopy and b. The length of the cells was measured (*n*=300) and the mean length (+/- one standard deviation) is shown. $p<0.001$ comparing length of cells harbouring pT7.5esaD(V584Y) in the presence and the absence of IPTG induction. or c. Cells were treated with the deadend fluorometric TUNEL system kit (Promega) and analysed by fluorescence light microscopy with a FITC filter. d. EsaD causes DNA damage. BL21(DE3) harbouring the indicated plasmids were cultured as in a., and cells were harvested prior to IPTG supplementation (0mM IPTG) and after three hour treatment with 1mM IPTG, deoxynucleotidyl transferase dUTP nick end labelled and fluorescence-positive cells quantified by flow cytometry. The percentage of total cells that are scored as fluorescence positive is given in the bottom left-hand corner of each panel.



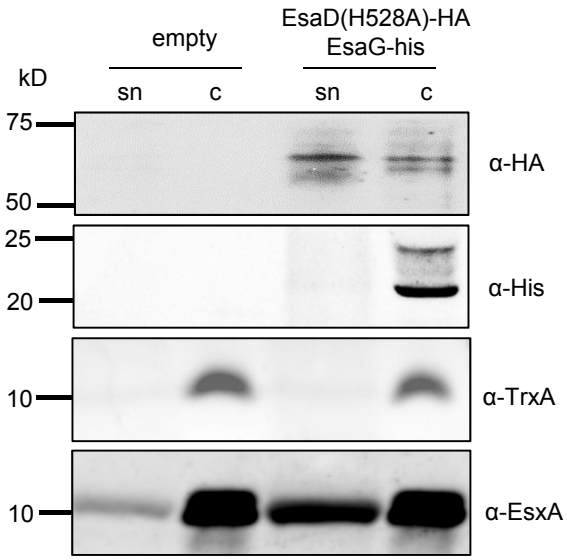
Supplementary Figure 4. Analysis of EsaD(H528A)-His-EsaG-HA, EsaD(H528A)-His-EsaE-HA and EsaD$_{421-614}$(H528A)-His-EsaG-HA co-purification by coomassie staining. The *S. aureus* wild type strain, RN6390, carrying EsaD(H528A)-His-EsaG-HA, pRAB11-EsaD(H528A)-His-EsaE-HA or pRAB11-EsaD(H528A)(421-614)-His-EsaG-HA was cultured in TSB medium to OD_{600} of 0.5 supplemented with ATC (500ng/ml) and harvested at OD_{600} of 3. Cells were lysed and histidine-tagged EsaD(H528A) was purified using nickel affinity beads. The cell lysate (load) and eluted fractions (20µl aliquots of each) were separated by SDS PAGE (12%) and stained with coomassie instant blue. The far left hand lane is the molecular weight marker used in the experiment. Note that these correspond to the same fractions used for western blotting in Fig 2c, Fig 2d and Fig 3b.



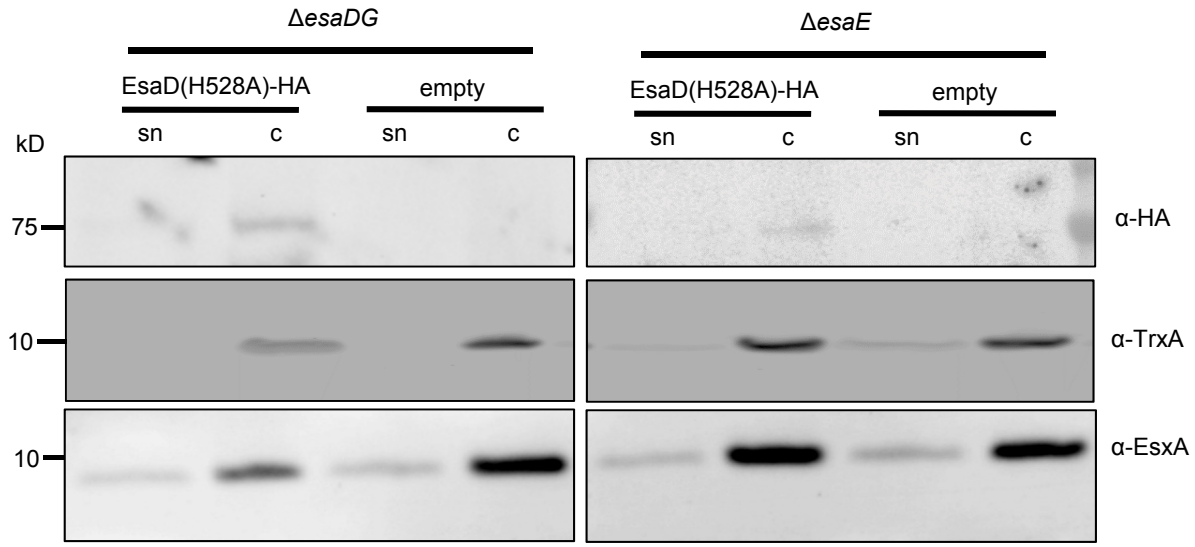
Supplementary Figure 5. EsaD is a Mg^{2+}-dependent DNase. 200ng of *S. aureus* gDNA or 400ng of a linear DNA PCR product were incubated with 0.4 µg purified $EsaD_{421-614}$-His, $EsaD_{421-614}$(H528A)-His or an equivalent volume of elution buffer, each supplemented with 50 mM $MgCl_2$ in a final volume of 20 µl at 37 °C for 20 mins after which the DNA was analysed by 1% agarose gel electrophoresis.



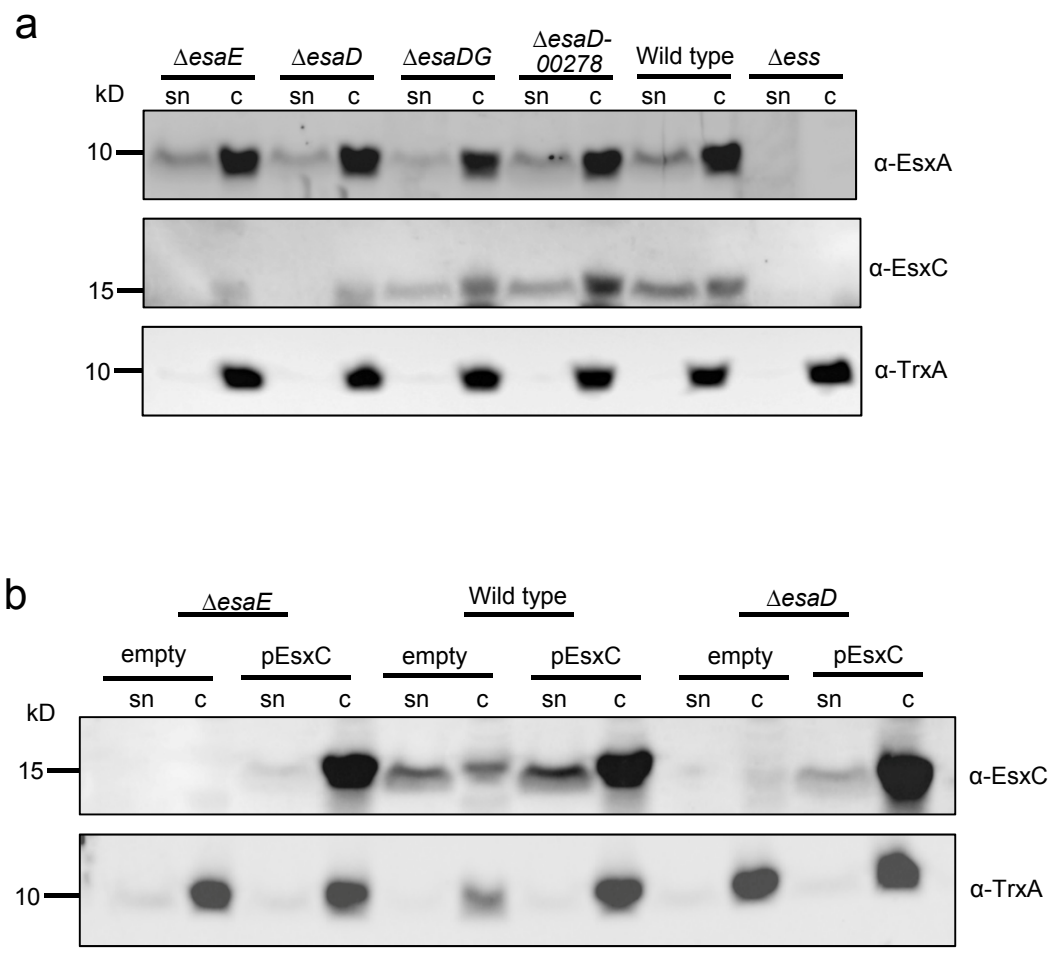
Supplementary Figure 6. EsaG is a cytoplasmic protein. The *S. aureus* wild type strain, RN6390, harbouring pRAB11 (empty) or pRAB11-EsaG-His was cultured in TSB medium to OD_{600} of 0.5, supplemented with ATC (250ng/ml) and harvested at OD_{600} of 2. A sample of the supernatant (sn) was retained as the secreted protein fraction and cells were fractionated into cell wall (cw), cytoplasmic (cyt) and membrane (m) fractions. Samples of each fraction (20μl aliquot of sn, cw, and cyt, 2mg of membrane) were separated on 12% bis-Tris gels and immunoblotted using either anti-His, anti-EssB (membrane protein control), anti-EsxA or anti-TrxA (cytoplasmic control) antisera.



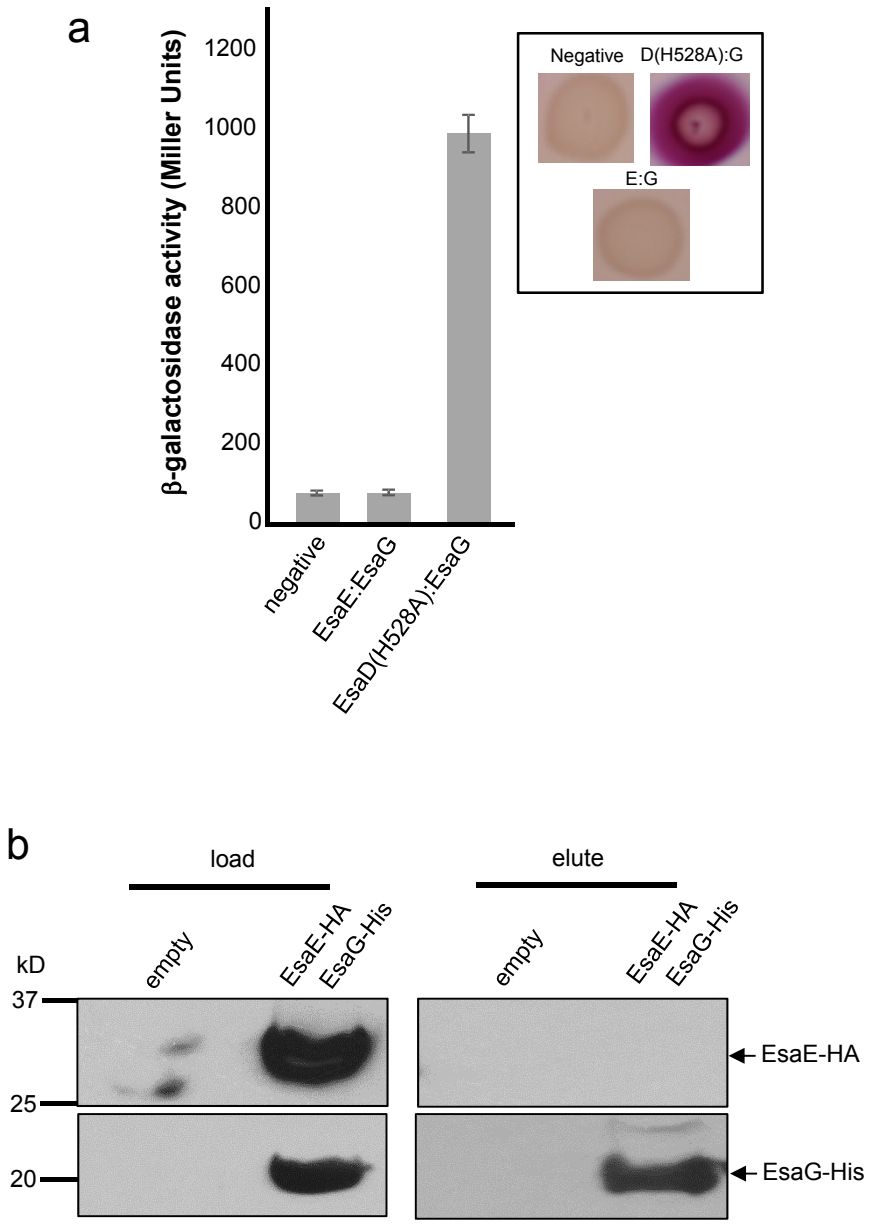
Supplementary Figure 7. EsaG is not co-secreted with EsaD. The *S. aureus* wild type strain, RN6390, harbouring pRAB11 (empty) or pRAB11-EsaD(H528A)-HA-EsaG-His were cultured in TSB medium to OD_{600} of 0.5, supplemented with ATC (250ng/ml) and harvested at OD_{600} of 2. Samples of the supernatant (sn) and cellular (c) fractions (an equivalent of 250μl of supernatant and 10μl of cells adjusted to OD_{600} of 1) were separated on 12% bis-Tris gels and immunoblotted using the indicated antisera.



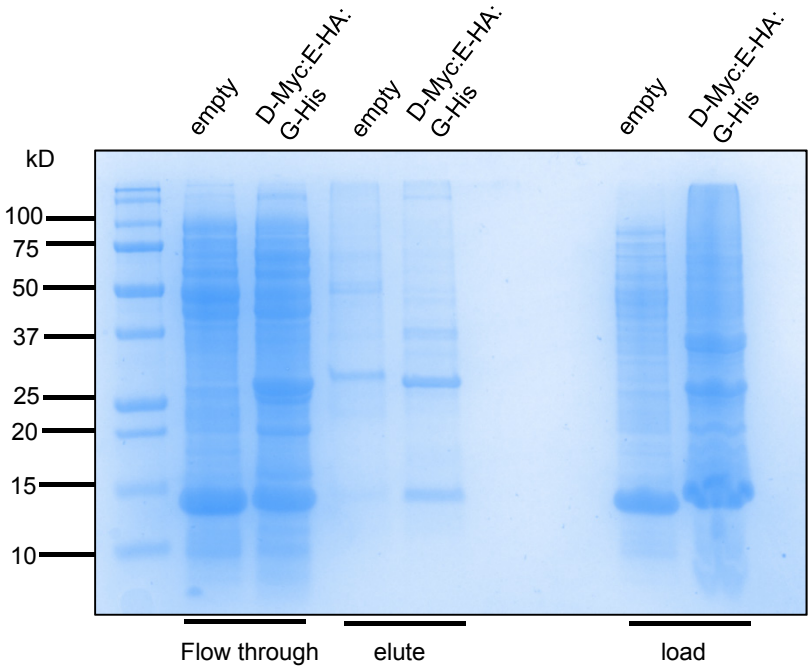
Supplementary Figure 8. EsaD-HA is not detected in the supernatant when *esaE* or *esaG* are deleted. The indicated *S. aureus* strains harbouring pRAB11 (empty) or pRAB11-EsaD(H528A)-HA were cultured in TSB medium until to OD_{600} of 0.5, supplemented with ATC (250ng/ml) and harvested at OD_{600} of 2. Samples of the supernatant (sn) and cellular (c) fractions (an equivalent of 250μl of supernatant and 10μl of cells adjusted to OD_{600} of 1) were separated on 12% bis-Tris gels and immunoblotted using the indicated antisera.



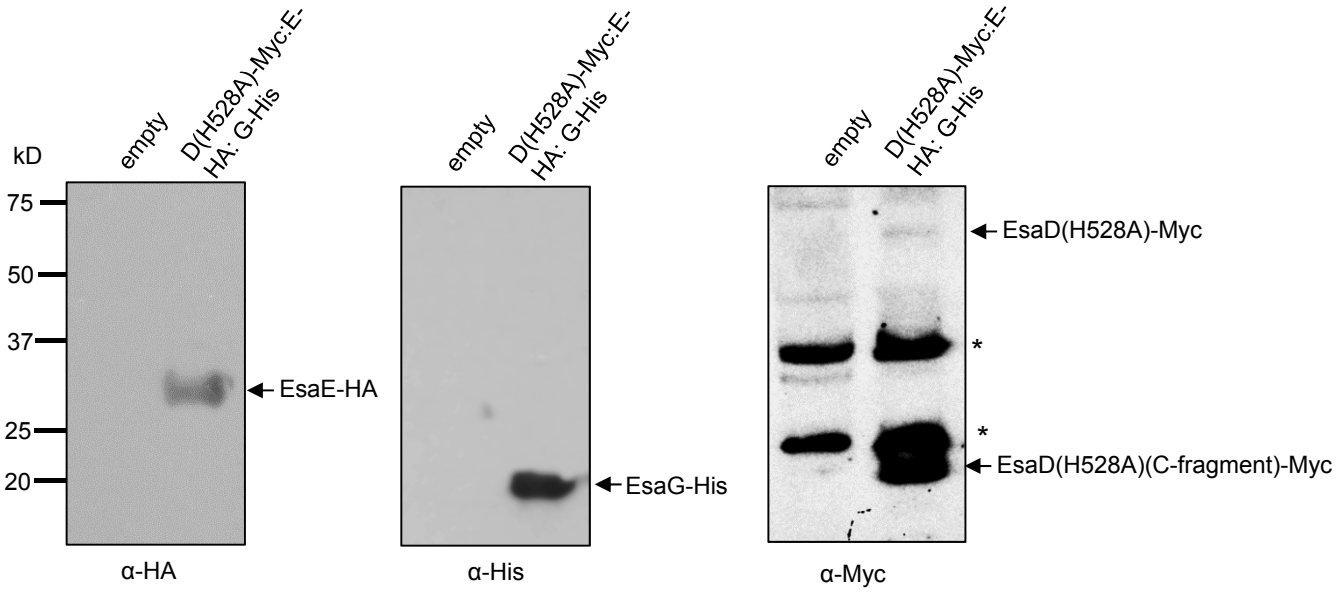
Supplementary Figure 9. EsaD and EsaG proteins are not required for the secretion of EsxA and EsxC. a. The RN6390 wild-type or isogenic deletion strains, as indicated, were cultured in TSB medium or b. The indicated strains harbouring pRAB11 (empty) or pRAB11-EsxC were cultured in TSB medium until an OD_{600} of 0.5 and supplemented with ATC (50ng/ml). a. and b. When cultures reached OD_{600} of 2, cells were spun down and the supernatant (sn) was retained as the secreted protein fraction, while the pellet was retained as the cellular fraction. Samples of the supernatant and cellular fractions (an equivalent of 200μl of supernatant and 10μl of cells adjusted to OD_{600} of 1) were separated on 12 % bis-Tris gels and immunoblotted with the indicated antisera.



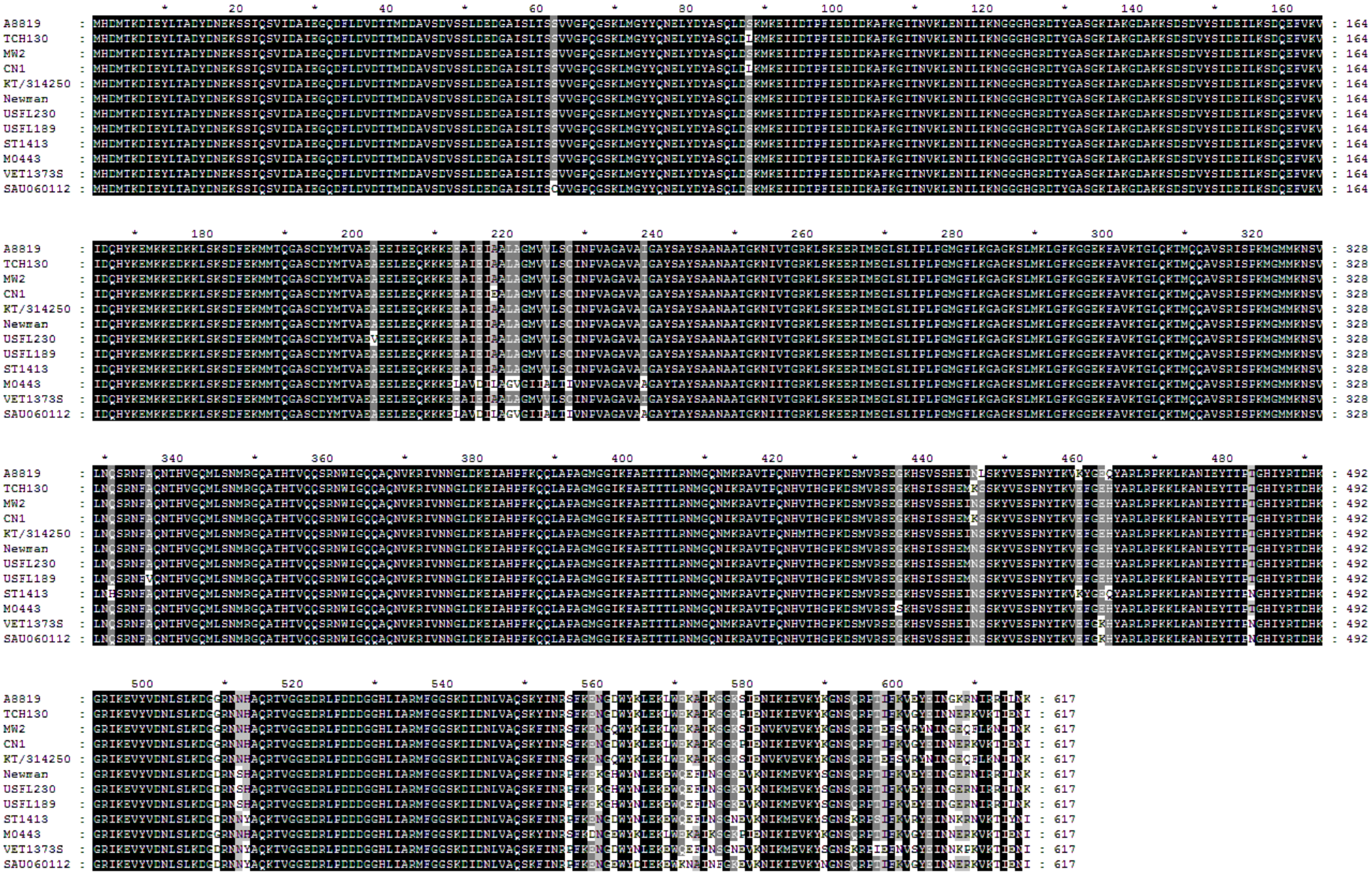
Supplementary Figure 10. EsaE and EsaG do not interact with each other. a. Bacterial two-hybrid analysis of strain BTH101 harbouring pT25 and pT18 (negative), pT25-EsaE and pT18-EsaG, or pT25-EsaG and pT18-EsaD(H528A) assessed by β-galactosidase activity assay. Error bars represent the standard deviation (*n*=3 biological replicates). The inset shows the same strain and plasmid combinations scored on MacConkey maltose plates. b. *E. coli* strain M15[pRep4] carrying pQE70 (empty) or pQE70-EsaE-HA-EsaG-His was cultured in LB medium to OD_{600} of 0.5, and then supplemented with 2 mM IPTG for 4 hours, after which cells were harvested and lysed. His-tagged EsaG was purified using Ni-affinity beads, and in each case the elution fractions (10μl aliquots of each) were separated by SDS PAGE (12%) and analysed by western blot with anti-HA or anti-His antibodies.



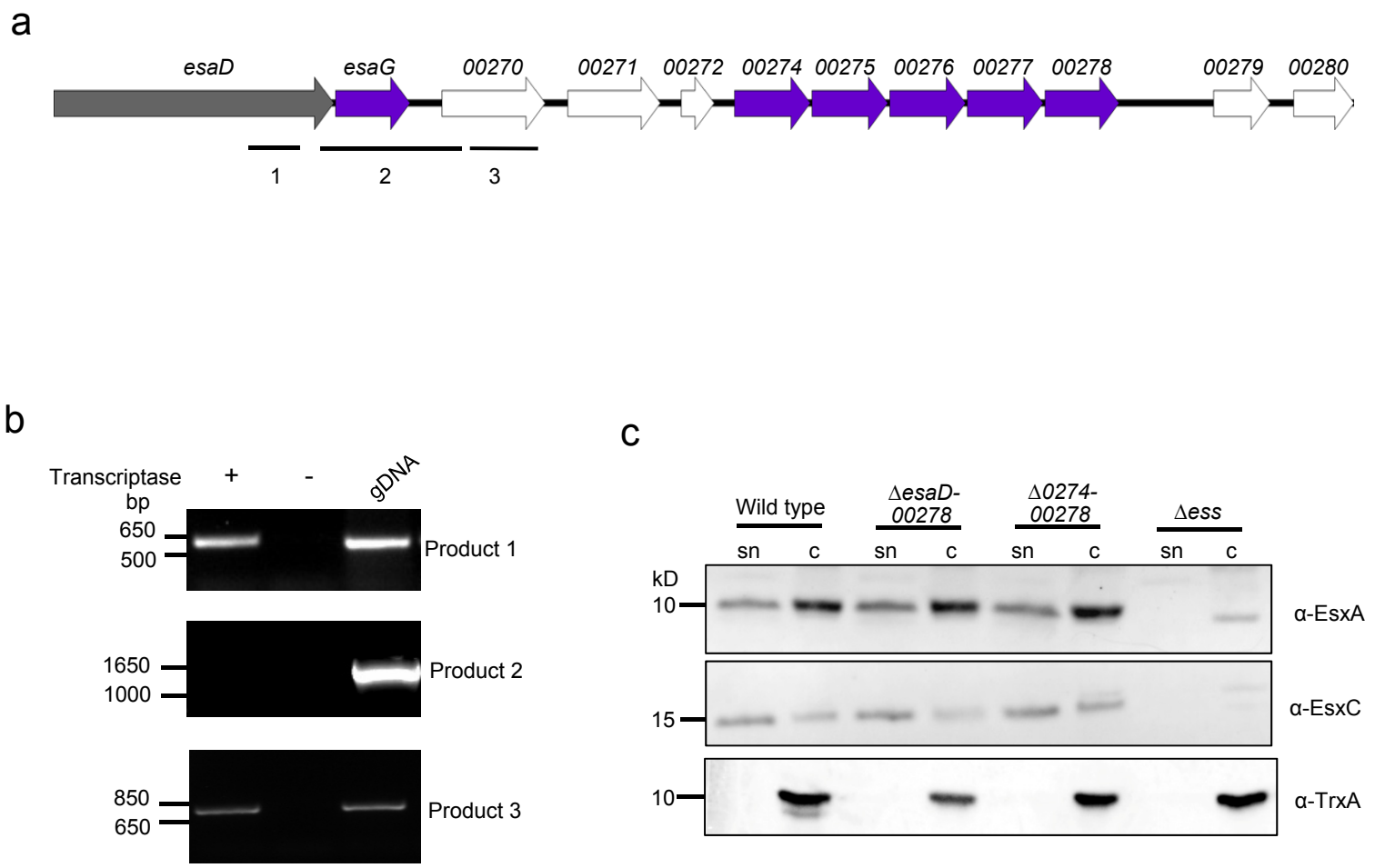
Supplementary Figure 11. Analysis of EsaE-HA-EsaD(H528A)-Myc-EsaG-His co-purification by coomassie staining. *E. coli* M15[pRep4] carrying pQE70 (empty) or pQE70- EsaE-HA-EsaD(H528A)-Myc-EsaG-His was cultured, lysed and EsaE-HA was purified using anti-HA antibody-coupled agarose beads as described in Methods and in the legend to Fig 3. The same samples (10μl aliquots of each) as those analysed in Fig 3f were stained using coomassie instant blue following SDS PAGE (12% bis-Tris gel). The far left hand lane is the molecular weight marker used in the experiment and the lanes between the load and elute fractions were left empty.



Supplementary Figure 12. EsaD, EsaE and EsaG form a ternary complex. *E. coli* strain M15[pRep4] carrying pQE70 (empty) or pQE70-EsaE-HA-EsaD(H528A)-Myc-EsaG-His was cultured in LB medium to OD_{600} of 0.5, and then supplemented with 2 mM IPTG for 4 hours, after which cells were harvested and lysed. His-tagged EsaG was purified using Ni-affinity beads, and 20μl aliquots the elution fractions (of each) were separated by SDS PAGE (12%) and analysed by western blot with the indicated antisera. Non-specific bands that cross-react with the anti-Myc antibody are indicated with asterisks.



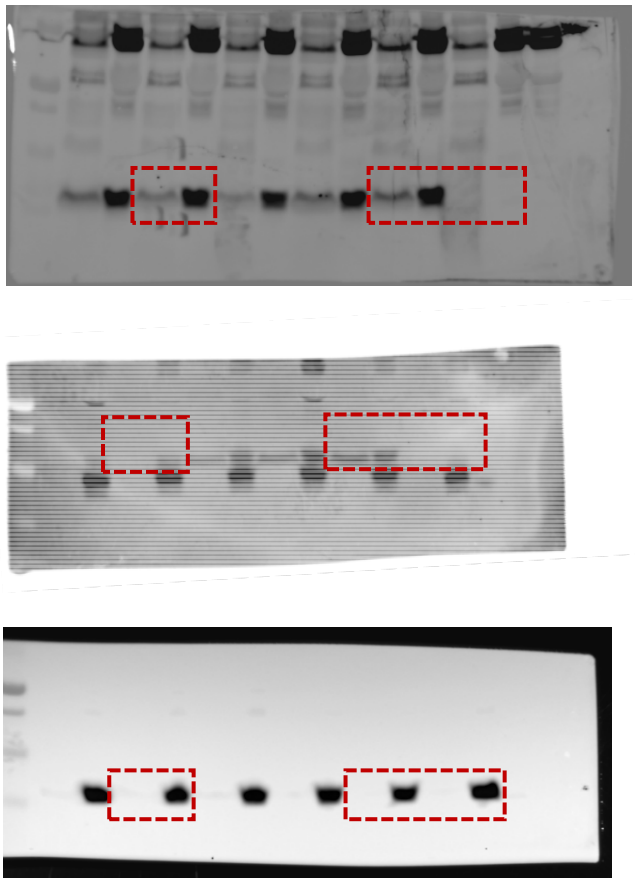
Supplementary Figure 13. Sequence analysis of EsaD proteins shows variability within the C-terminal nuclease domain. EsaD protein sequences from *S. aureus* subsp. *aureus* strains were extracted from the KEGG Genome database (www.genome.jp/kegg/genome.html) and aligned using Clustal Omega on standard settings. Redundant sequences (100% identity) were omitted for clarity.



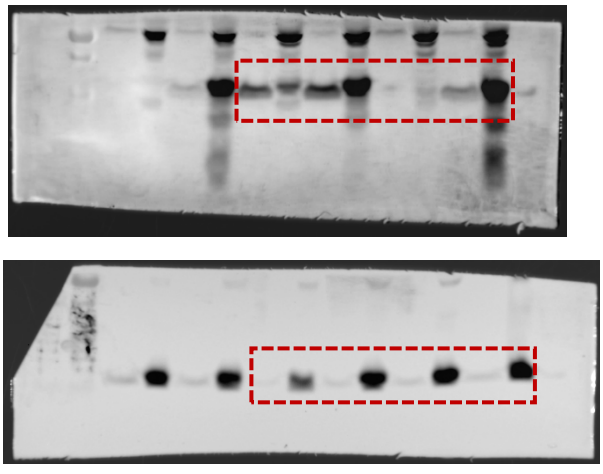
Supplementary Figure 14. Genes encoding additional DUF600 domain proteins are not co-transcribed with *esaG* and are not required for EsxA and EsxC secretion. a. Genetic organisation at the 3' end of the *ess* locus in S. aureus strain NCTC8325, with genes encoding DUF600 proteins are shaded in purple. The regions amplified by primer pairs used for RT-PCR analysis (listed in Table S3) are indicated. b. RT-PCR analysis of mRNA isolated from *S. aureus* strain RN6390, using primer pairs. The expected sizes for PCR products 1-3 are 573, 1143 and 684 bp, respectively. c. The RN6390 wild-type or isogenic deletion strains, as indicated, were cultured in TSB medium to OD600 of 2, cells (c) were harvested and the supernatant (sn) was retained as the secreted protein fraction. Samples of the supernatant and cellular fractions (an equivalent of 250μl of supernatant and 10 μl of cells adjusted to OD_{600} of 1) were separated on 12% bis-Tris gels and immunoblotted using either anti-EsxA, anti-EsxC or anti-TrxA antisera.

Supplementary Figure 15. Uncropped images of Figs. 1-4 and Supplementary Figs. 5-10, 12 and 14.

Uncropped Figure 1b



Uncropped Figure 1c



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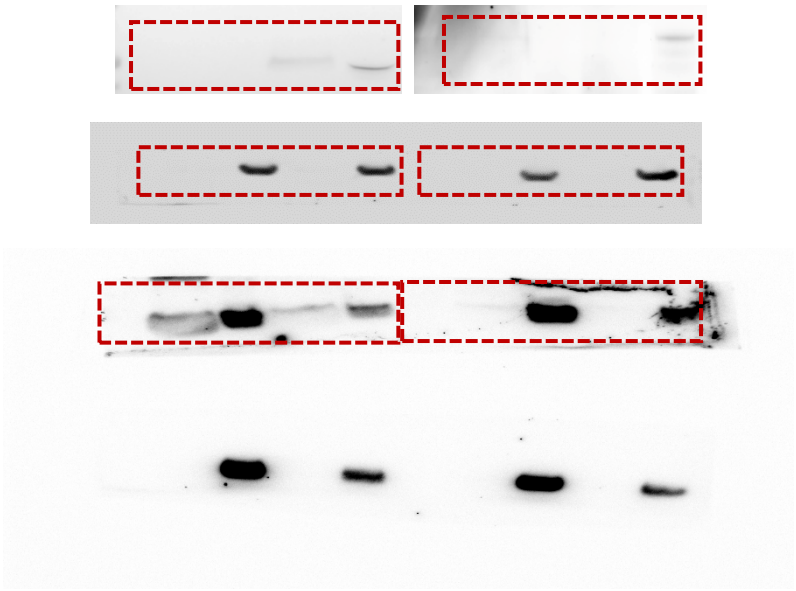

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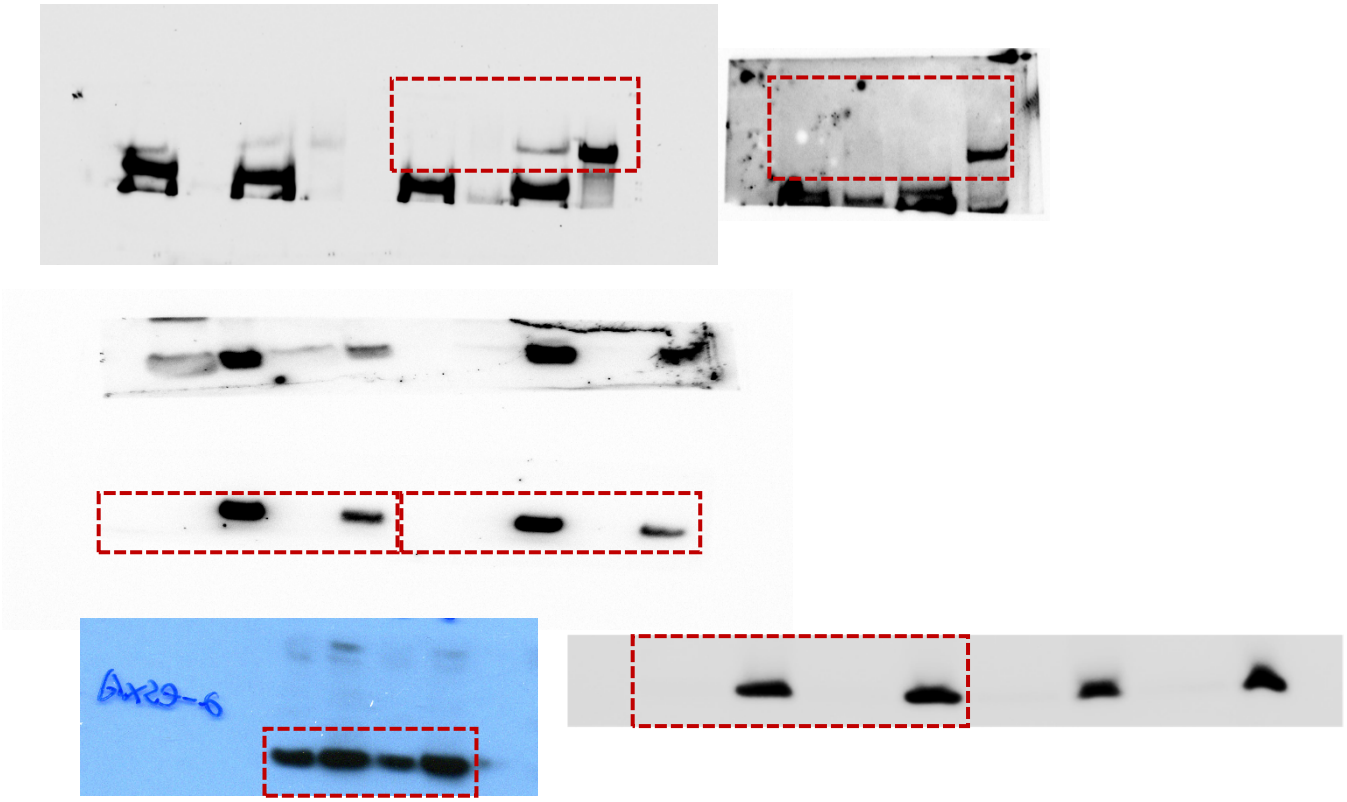

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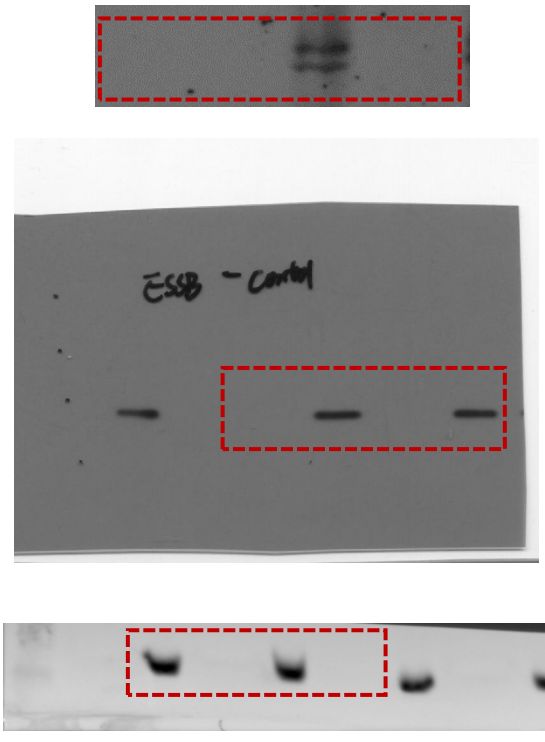

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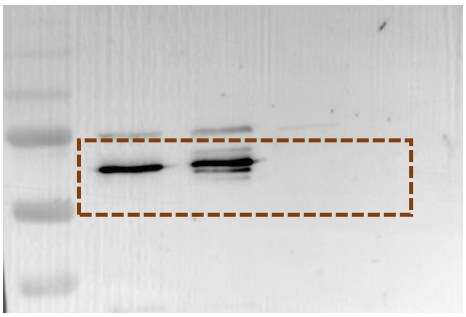

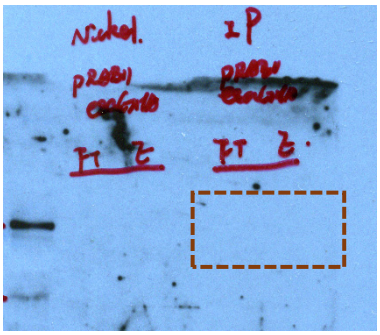

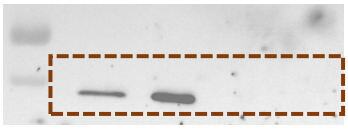

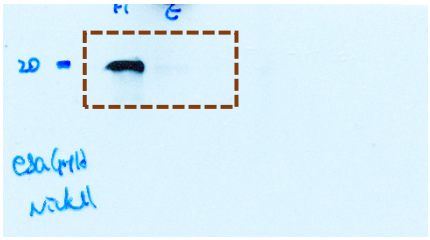

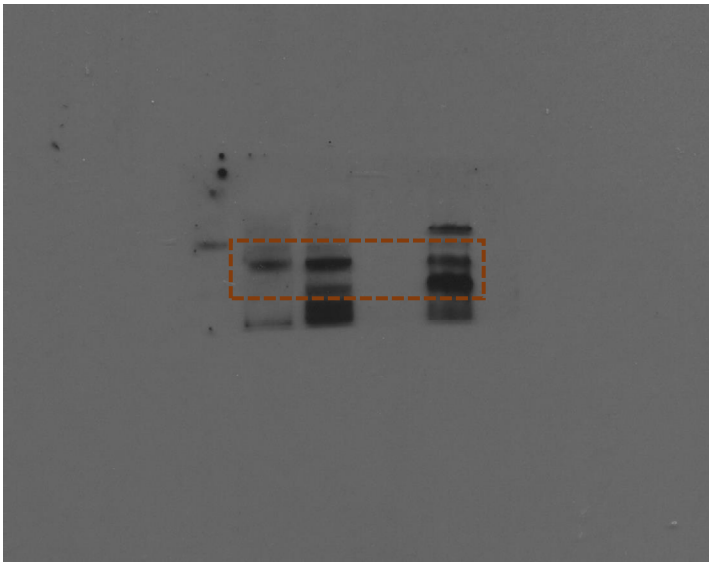

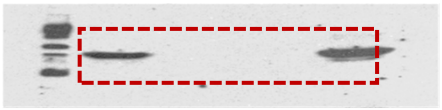

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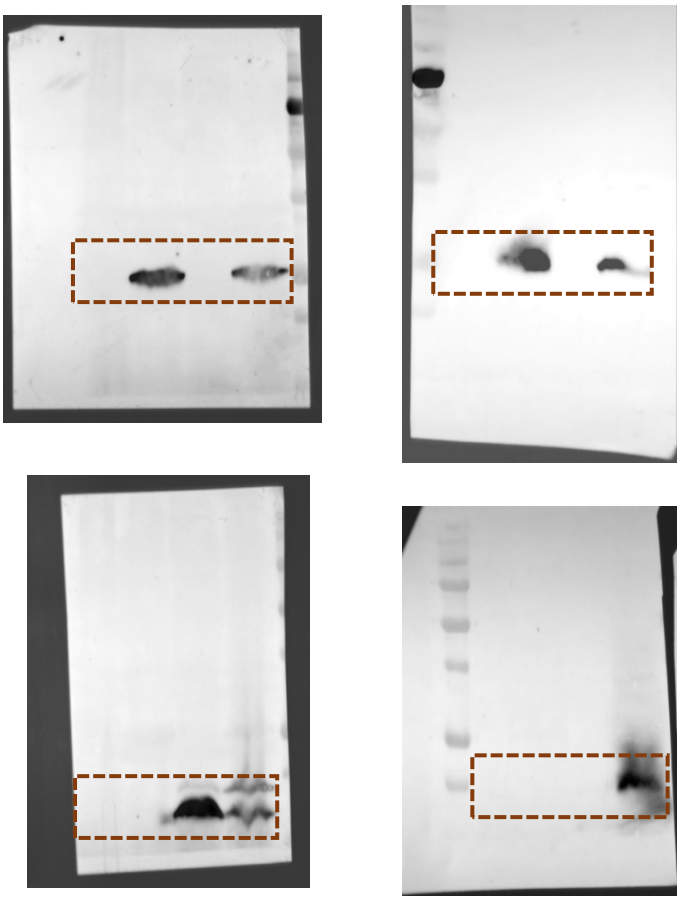

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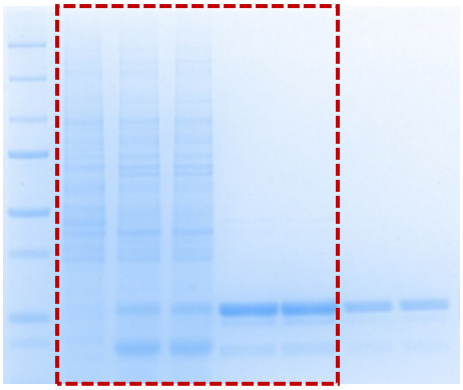

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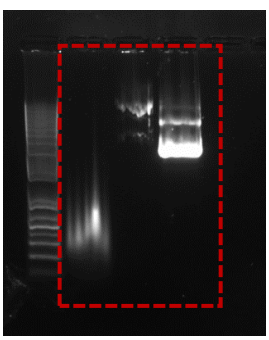

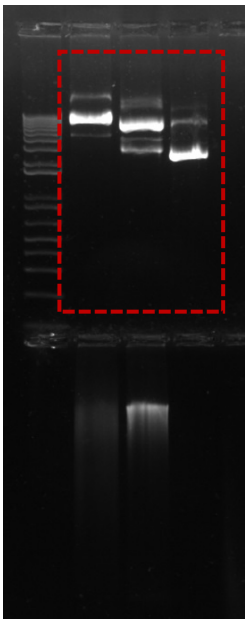

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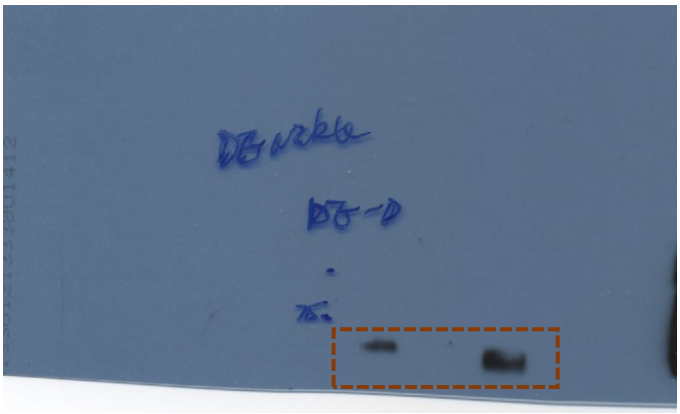

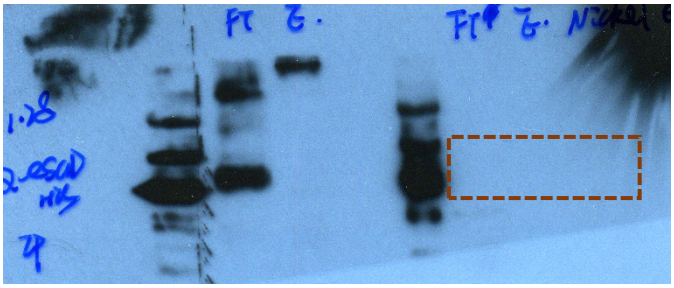

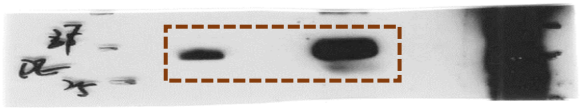

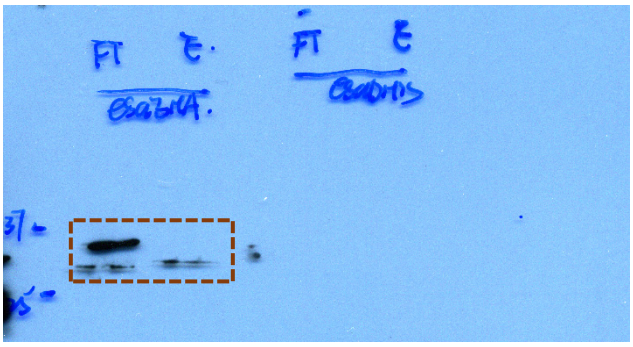

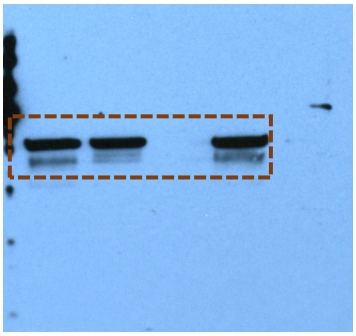

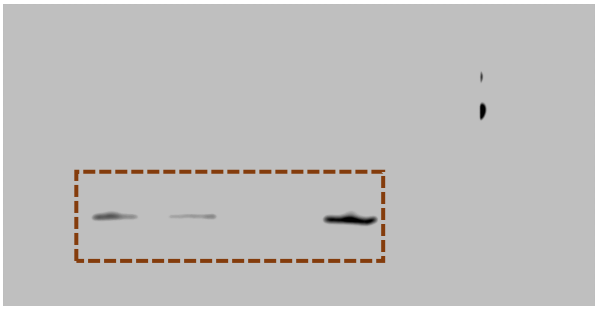

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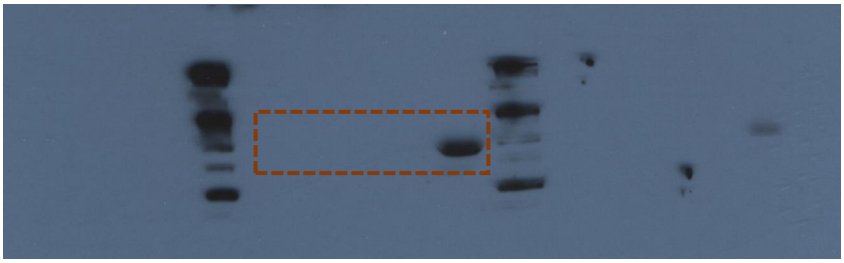

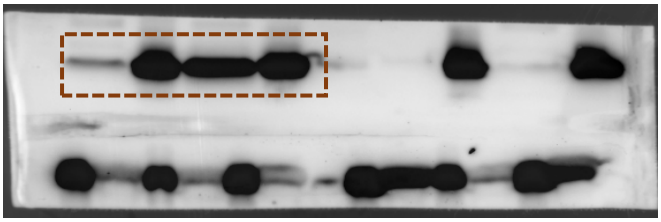

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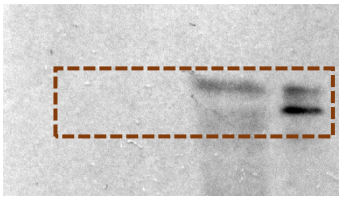

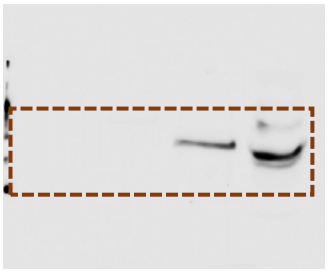

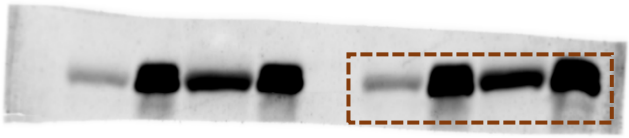

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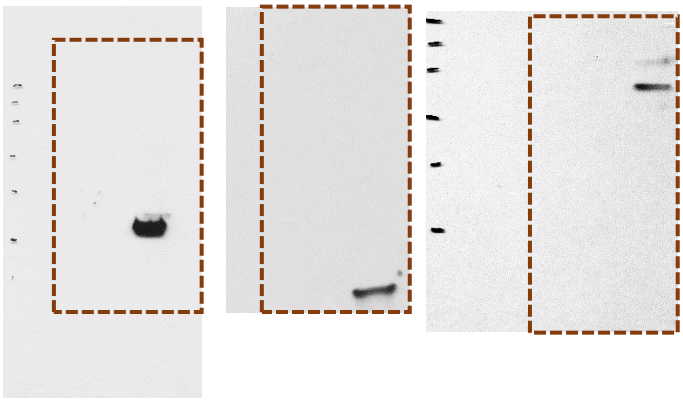

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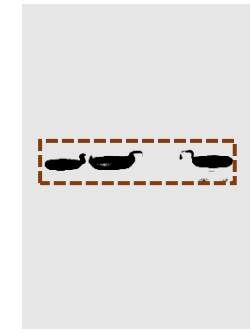
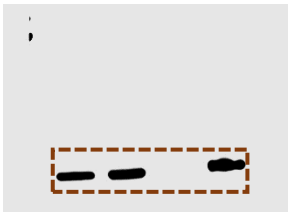
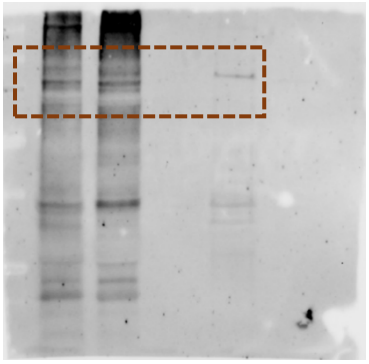
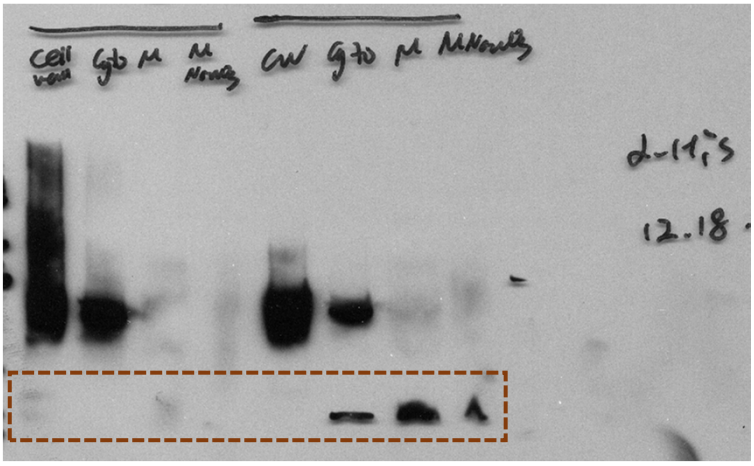
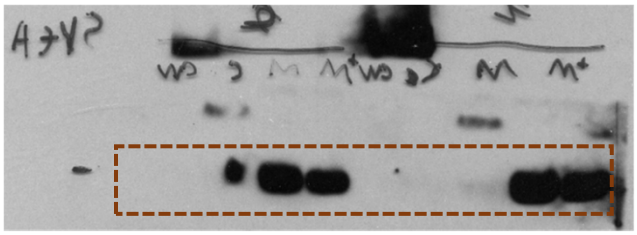






Uncropped Figure 4a





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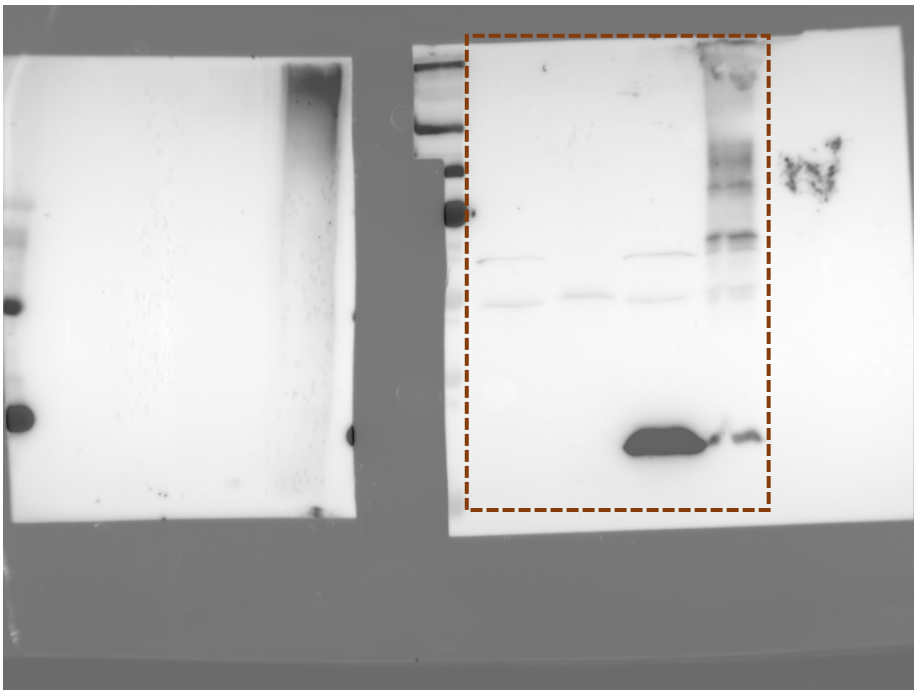

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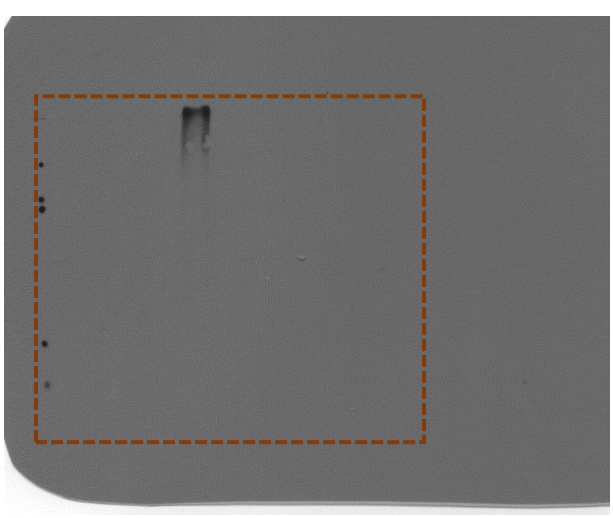

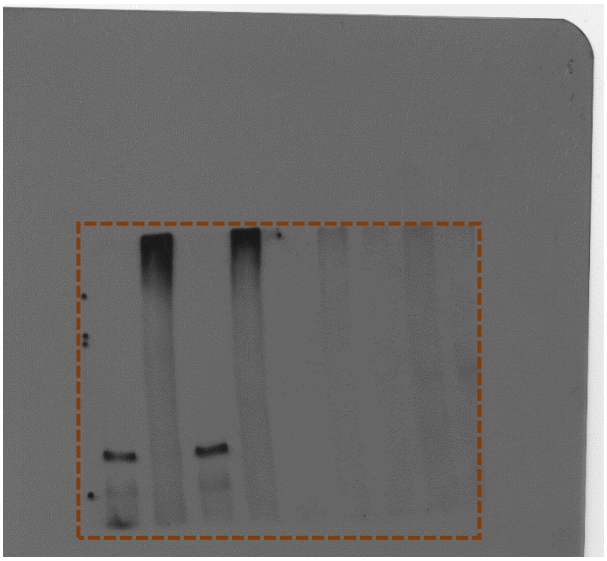

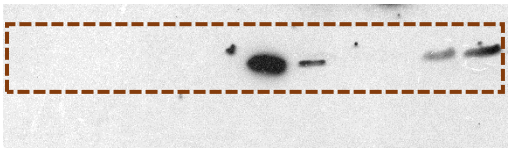

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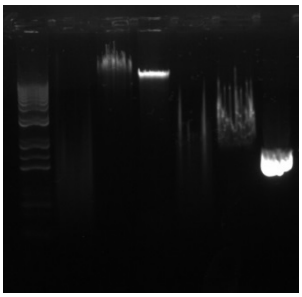

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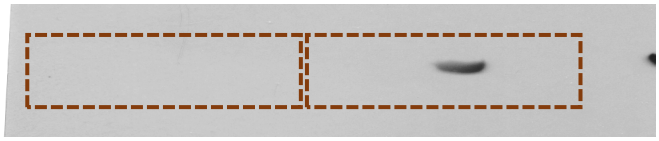

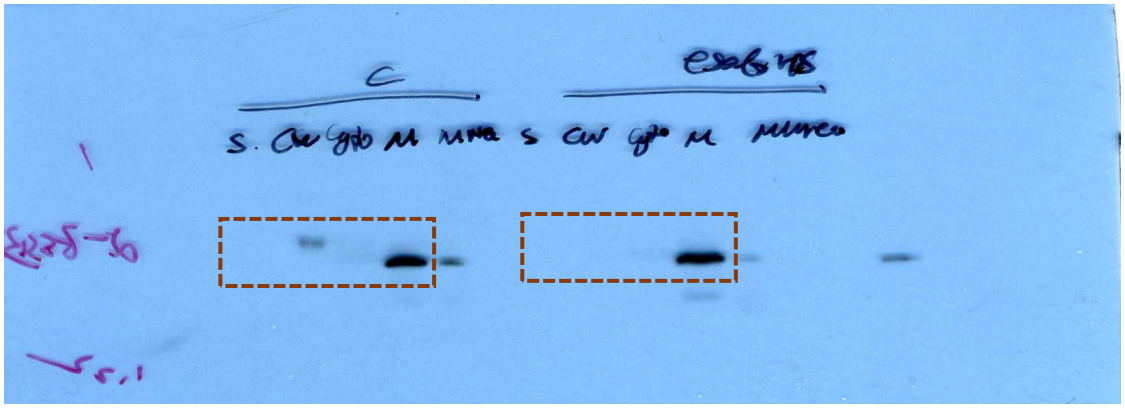

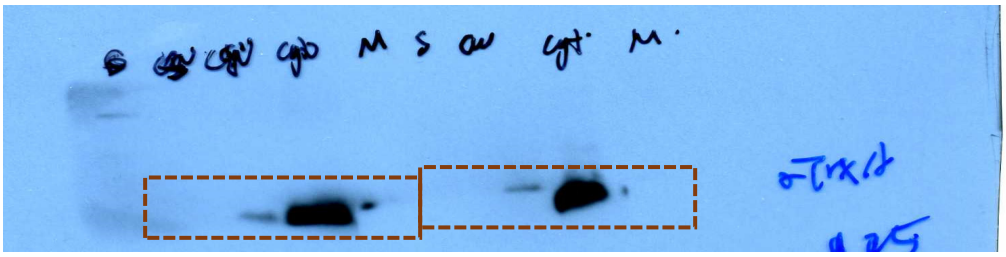

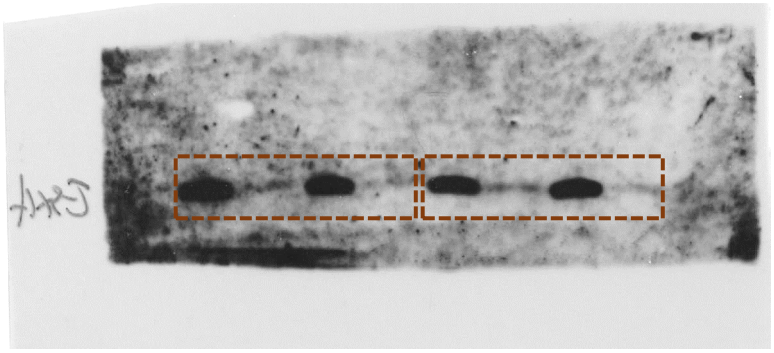

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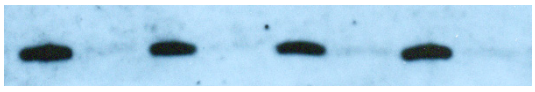
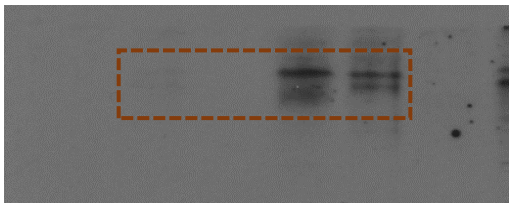
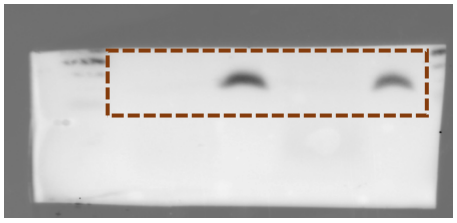
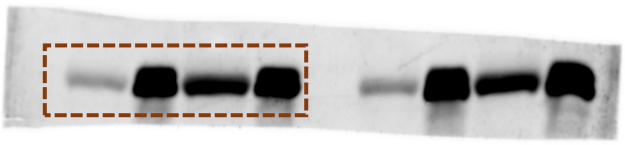


Uncropped Supplementary Figure 7







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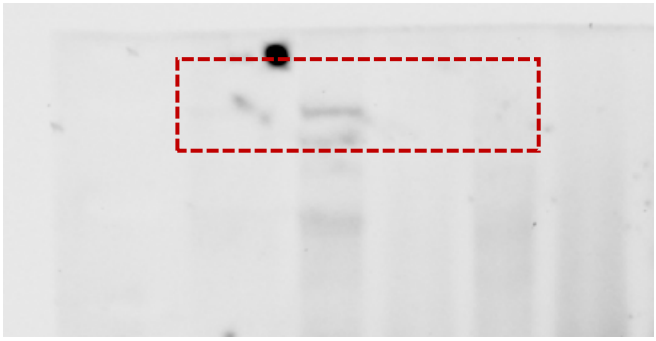
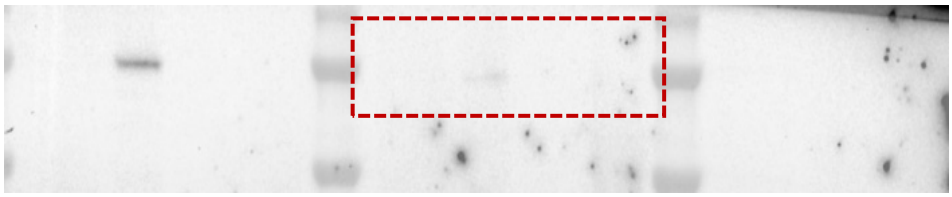
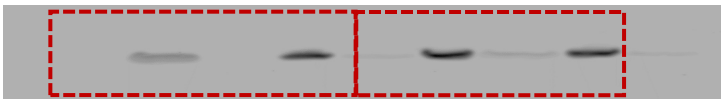
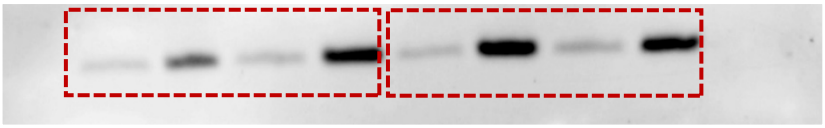
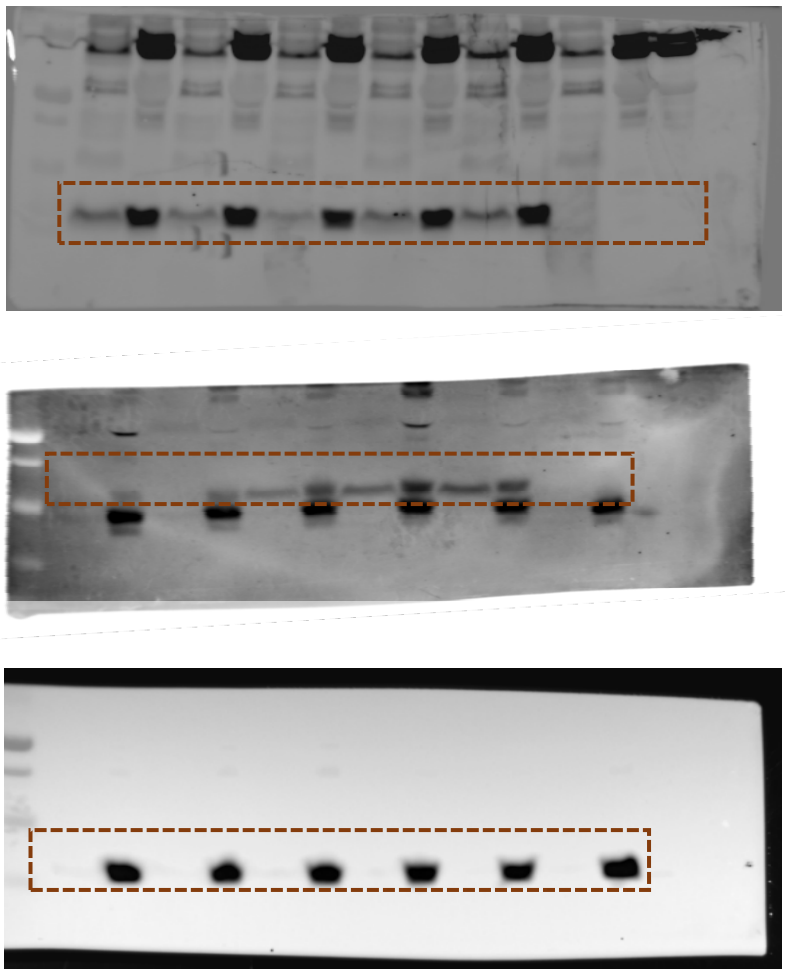




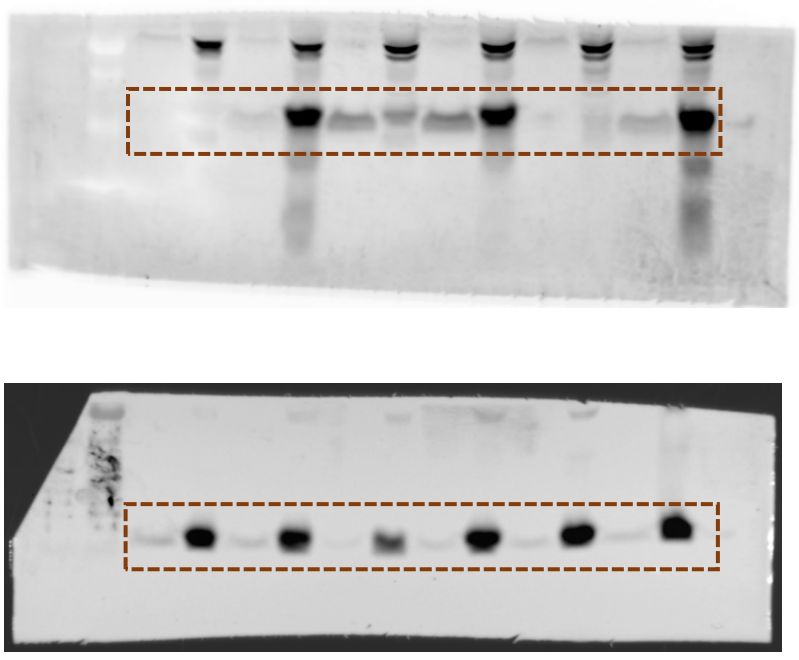




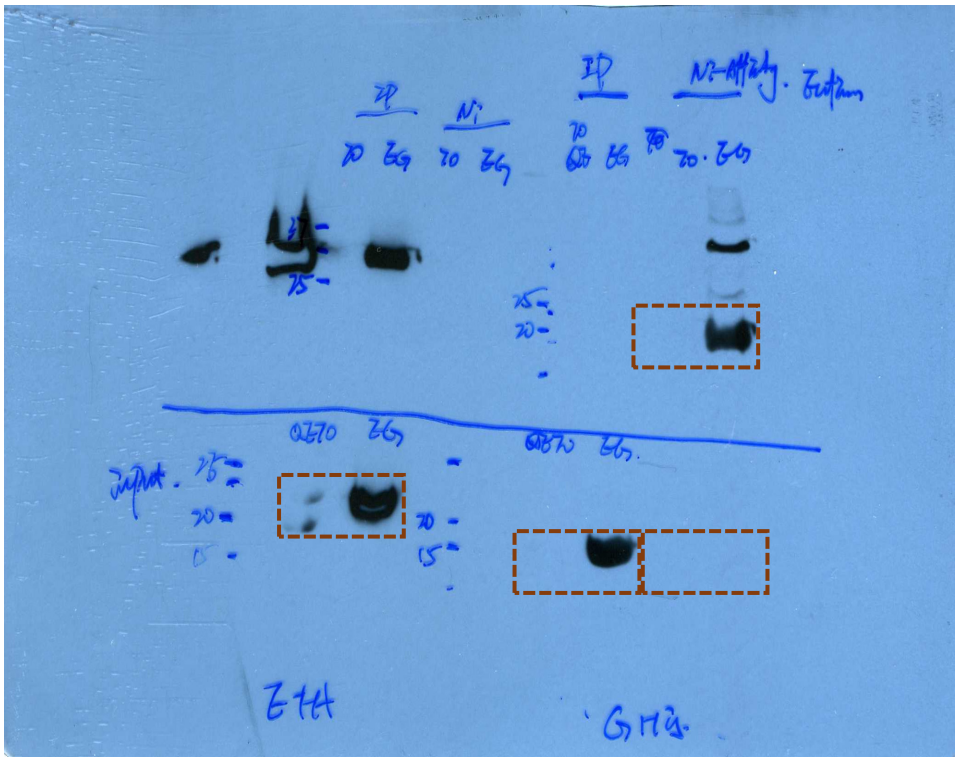
Uncropped Supplementary Figure 9a



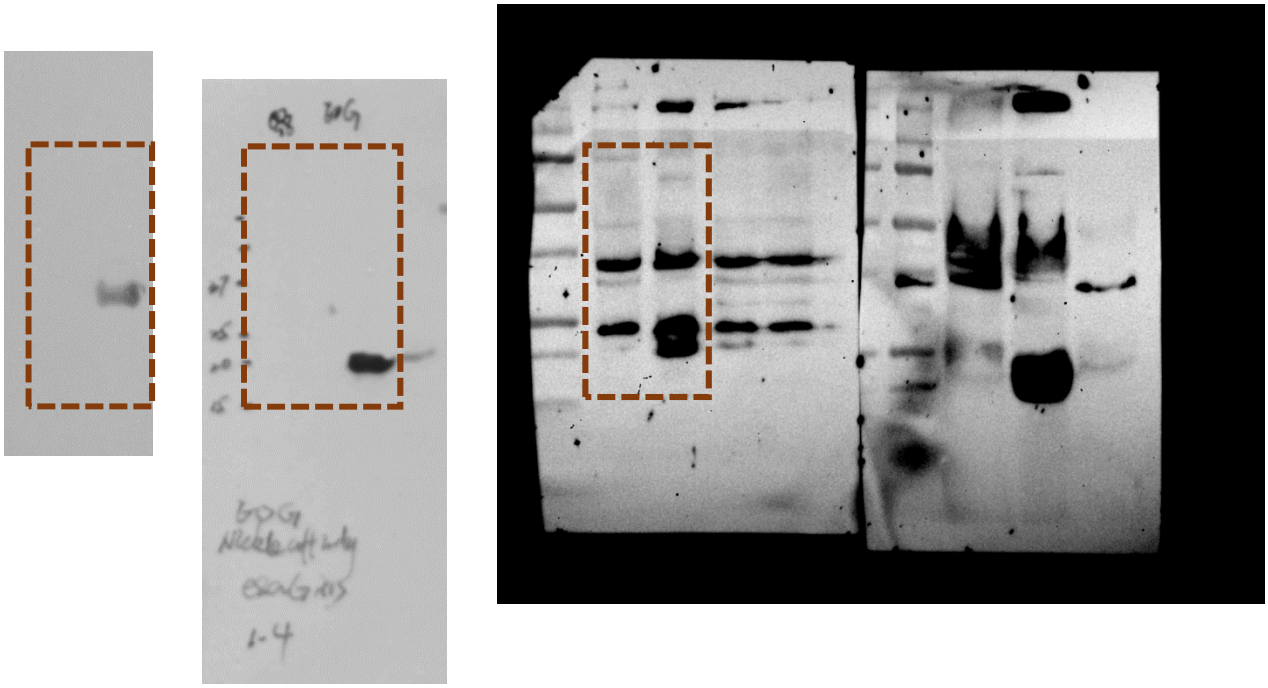
Uncropped Supplementary Figure 9b



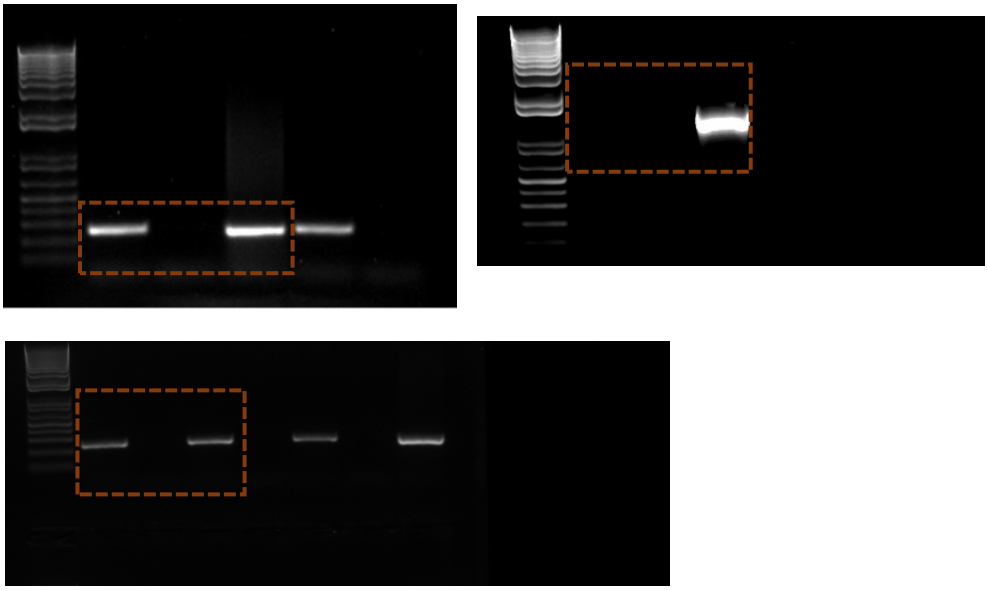
Uncropped Supplementary Figure 10



Uncropped Supplementary Figure 12



Uncropped Supplementary Figure 14b



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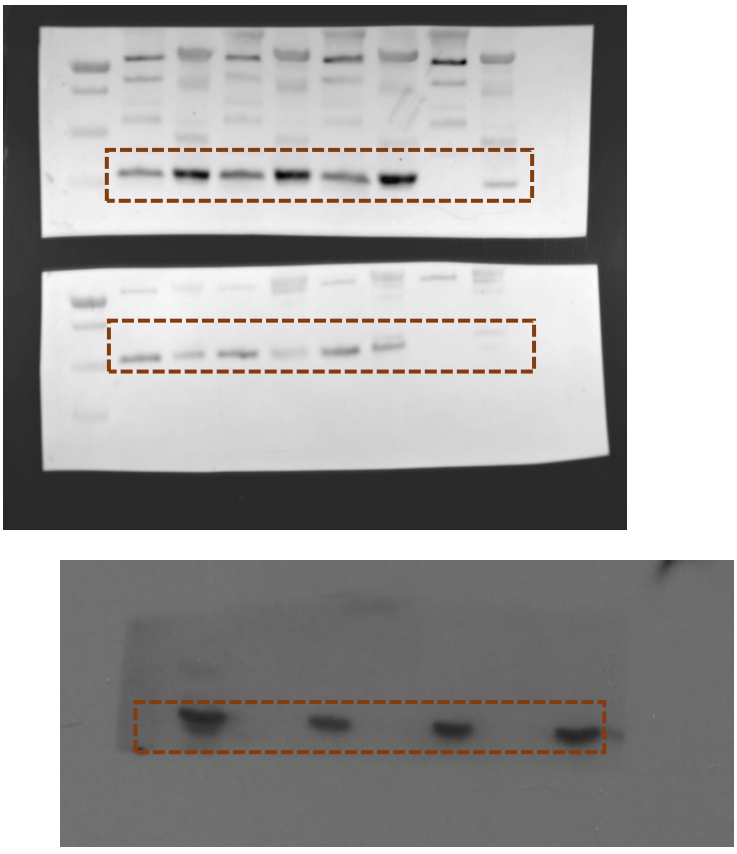

Supplementary Figure 16. Synthetic gene sequences used in this study.

Sequence of insert in pQE70 EsaE-HA-EsaD-Myc-EsaG-His

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GTATCAACGACATGTACTTCGCGCCGTTCTCTTACGAAAAAGACGAATACATCCTGCTG
TCTCGTTTCAAAAACAACGGTTTCCAGATCCGTATCATCAACAAAGACATCGCGTGGTG
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*ACTGGAA*AGATCT

Restriction sites are underlined, genes (*esaE, esaD* and *esaG*) are shown in italics and added tags (HA, Myc) are double underlined. Note that the C-terminal His-tag on EsaG is supplied by the vector.

Synthetic pT25-DUF600 constructs

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Restriction sites are underlined

***ermC* integration synthetic construct**

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GGTGTTGGTATTTCATTTGGTATTGTGGCTTCGTCTCGAATTC

Restriction sites underlined, *ermC* sequence in italics, *rpsF* promoter sequence from *B. subtilis* and *trxA* rho-independent terminator sequence from *S. aureus* flanking the *ermC* gene are dashed and the whole integration cassette sandwiched between the homology region (622 bp 5', 617 bp 3') for integration.

Synthetic *ess* region from *esxA* to *esxB*, codon optimised for *E. coli*.

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Restriction sites underlined, start and stop codons shown in upper case.

Supplementary Table 1. Strains used in this study

Strain	Relevant genotype or description	Source or reference
S. aureus strains		
RN6390	NCTC8325 derivative, *rbsU*, *tcaR*, cured of φ11, φ12, φ13	Refence[1]
Δ*essC*	As RN6390, Δ*essC*	Reference[2]
Δ*ess*	Complete deletion from *esxA* – *esaG*	Reference[2]
Δ*esaE*	As RN6390, Δ*esaD*	This work
Δ*esaD*	As RN6390, Δ*esaE*	This work
Δ*esaDG*	As RN6390, Δ*esaDG*	This work
Δ*saouhsc00268-00278*	As RN6390, Δ*esaD-saouhsc00278*	This work
Δ*saouhsc00274-00278*	As RN6390, Δ*saouhsc00274-saouhsc00278*	This work
RN6390*::ermC*	As RN6390, with *ermC* resistance gene chromosomal insertion	This work
Δ*esaDG::ermC*	As Δ*esaDG* with *ermC* resistance gene from RN6390*::ermC* (phage ϕ11 transduction)	This work
Δ*saouhsc00268-00278::ermC*	As Δ*esaD-saouhsc00278* with *ermC* resistance gene from RN6390*::ermC* (phage ϕ11 transduction)	This work
Δ*saouhsc00274-00278::ermC*	As Δ*saouhsc00274-saouhsc00278* with *ermC* resistance gene from RN6390*::ermC* (phage ϕ11 transduction)	This work
COL	MRSA, *agr*	Reference[3]
COLΔ*ess*	Complete deletion from *Sacol0271* (*esxA*) - *Sacol0282*	Reference[2]
E. coli strains		
JM110	*rpsL thr leu thi lacY galK galT ara tonA tsx dam dcm glnV44 Δ(lac-proAB)* e14- [F' *traD36 proAB*$^{+}$ *lacI*^{q} *lacZ*ΔM15] *hsdR17*(rK^{-}mK^{+})	Stratagene
BL21(DE3)	*E. coli* B: F^{-}, *dcm*, *ompT*, *hsdS*(rB-, mB-), *gal*, λDE3	Reference[4]

M15 [pREP4]	*F*-, *lac*, *ara*, *gal*, *mtl* [(*Kan*^{R}, *lacI*]	Qiagen
BTH101	F^{-} *cya-99, araD139, galE15, galK16, rpsL1 (*Str^{r}*), hsdR2, mcrA1, mcrB1*	Reference[5]

Supplementary Table 2. Plasmids used in this study

Plasmid	Relevant genotype or description	Source or reference
pIMAY	*E. coli/S. aureus* shuttle vector, temperature sensitive, cml^r	[6]
pIMAY-esaE	pIMAY carrying *esaE* deletion allele	This work
pIMAY-esaD	pIMAY carrying *esaD* deletion allele	This work
pIMAY-esaDG	pIMAY carrying *esaDG* deletion allele	This work
pIMAY-saouhsc00268-00278	pIMAY carrying *esaD-saouhsc00278* deletion allele	This work
pIMAY-saouhsc00274-00278	pIMAY carrying *saouhsc00274-saouhsc00278* deletion allele	This work
pRAB11	*E. coli/S. aureus* shuttle vector, inducible protein expression, amp^r, cml^r	Reference[7]
pRAB11-EsaD(H528A)-HA	pRAB11 producing C-terminally HA-tagged EsaD	This work
pRAB11-EsaE-his	pRAB11 producing C-terminally His-tagged EsaE	This work
pRAB11-EsaG-his	pRAB11 producing C-terminally His-tagged EsaG	This work
pRAB11-EsaD-His-EsaG-HA	pRAB11 producing C-terminally His-tagged EsaD and C-terminally HA-tagged EsaG	This work
pRAB11-EsaD-HA-EsaG-His	pRAB11 producing C-terminally HA-tagged EsaD and C-terminally His-tagged EsaG	This work
pRAB11-EsaD(H528A)-His	pRAB11 producing C-terminally His-tagged H528A substituted EsaD	This work
pRAB11-EsaG-HA	pRAB11 producing C-terminally HA-tagged EsaG	This work
pRAB11-EsaE-HA	pRAB11 producing C-terminally HA-tagged EsaE	This work
pRAB11-EsaD(H528A)-His-EsaE-HA	pRAB11 producing C-terminally His-tagged H528A substituted EsaD and C-terminally HA-tagged EsaE	This work
pRAB11-EsaD(H528A)-HA-EsaE-His	pRAB11 producing C-terminally HA-tagged H528A-substituted EsaD and C-terminally His-tagged EsaE	This work
pRAB11-$EsaD_{421-614}$ (H528A)-His-EsaG-HA	pRAB11 producing C-terminally His-tagged H528A substituted nuclease domain of EsaD (aa 421-614and C-terminally HA-tagged EsaG	This work
pRAB11-$EsaD_{421-614}$ (H528A)-His	pRAB11 producing C-terminally His-tagged H528A substituted nuclease domain of EsaD (aa 421-614)	This work
pRAB11-EsxC	pRAB11 producing native (untagged) EsxC	This work
pQE70	Vector for regulatable protein overproduction in *E. coli* (T5 promoter). Amp^r	Qiagen
pQE70-EsaE-HA-EsaD-Myc-EsaG-His[1]	pQE70 producing C-terminally HA-tagged EsaE, C-terminally Myc-tagged EsaD and C-terminally His-tagged EsaG	This work

pQE70-EsaE-HA-EsaD(H528A)-Myc-EsaG-His†	pQE70 producing C-terminally HA-tagged EsaE, C-terminally Myc-tagged H528A-substituted EsaD and C-terminally His-tagged EsaG	This work
pQE70-EsaE-HA-EsaG-His	pQE70 producing C-terminally HA-tagged EsaE and C-terminally His-tagged EsaG	This work
pQE70-EsaG-EsaG-$EsaD_{421-614}$-His	pQE70 producing tandem copies of EsaG and C-terminally His-tagged $EsaD_{421-614}$	This work
pQE70-EsaG-EsaG-$EsaD_{421-614}$(H528A)-His	pQE70 producing tandem copies of EsaG and C-terminally His-tagged H528A substituted $EsaD_{421-614}$	This work
pT7.5	Vector for regulatable protein production in *E. coli* (T7 promoter). Amp^r	Reference[8]
pT7.5esaD(V584Y)	pT7.5 producing V584Y substituted EsaD	This work
pT7.5esaDG	pT7.5 producing EsaD and EsaG	This work
pT18	Vector encoding T18 fragment of *B. pertussis cyaA*; Amp^r	Reference[9]
pT18-EsaE	pT18 carrying *esaE*	This work
pT18-EsaD(H528A)	pT18 carrying *esaD* (H528A codon substitution)	This work
pT18-$EsaD_{1-420}$	pT18 carrying *esaD* (codons 1-420)	This work
pT18-EsaG	pT18 carrying *esaG*	This work
pT25	Vector encoding T18 fragment of *B. pertussis cyaA*; cml^r	Reference[9]
pT25-EsxA	pT25 carrying *esxA*	This work
pT25-EsxB	pT25 carrying *esxB*	This work
pT25-EsaB	pT25 carrying *esaB*	This work
pT25-EsxC	pT25 carrying *esxC*	This work
pT25-EsaE	pT25 carrying *esaE*	This work
pT25-EsaD(H528A)	pT25 carrying *esaD* (H528A codon substitution)	This work
pT25-$EsaD_{421-614}$	pT25 carrying *esaD* (codons 421-614, with H528A codon substitution)	This work
pT25-EsxD	pT25 carrying *esxD*	This work
pT25-EsaG	pT25 carrying *esaG*	This work
pT25-saouhsc00274*	pT25 carrying *saouhsc00274*	This work
pT25-saouhsc00275*	pT25 carrying *saouhsc00275*	This work
pT25-saouhsc00276*	pT25 carrying *saouhsc00276*	This work

pT25-saouhsc00277*	pT25 carrying *saouhsc00277*	This work
pT25-saouhsc00278*	pT25 carrying *saouhsc00278*	This work
pT25-SAPIG0310*	pT25 carrying *SAPIG0310*	This work
pT25-SAPIG0311*	pT25 carrying *SAPIG0311*	This work
pT25-SAPIG0314*	pT25 carrying *SAPIG0314*	This work
pT25-SAR0293*	pT25 carrying *SAR0293*	This work
pT25-SAR0294*	pT25 carrying *SAR0294*	This work
pT25-SAR0295*	pT25 carrying *SAR0295*	This work
pT25-SAR0297*	pT25 carrying *SAR0297*	This work
pT25-SAEMRSA15_02570*	pT25 carrying *SAEMRSA15_02570*	This work
pT25-SAEMRSA15_02580*	pT25 carrying *SAEMRSA15_02580*	This work

†Purchased ready-cloned in the pQE70 vector from Biomatik.

*Purchased ready-cloned in the pT25 vector from Genescript.

Supplementary Table 3. Oligonucleotides and cloning strategies used in this study

Name	Nucleotide Sequence (5'-3')	Template	Restriction Enzyme	Usage
EsaE-for-bglII-esxA-RBS	GCAGATCTAGGAGGTTTCTACTTATGAAAGATGTT AAGCGAAT	gDNA	*Bgl*II	Construction of pRAB11-EsaE-his[1] and pRAB11-EsaE-HA[1]
EasE-rev-sacI-HA	GCGAGCTCTTATGCATAATCTGGAACATCATATGGATACTCCT CTGCTTTATTAATATGAT	gDNA	*Sac*I	Construction of pRAB11-EsaE-HA[1]
EasE-rev-sacI-His	GCGAGCTCTTAGTGGTGGTGGTGGTGGTGCTCCTCTGCTTTATTA ATATGAT	gDNA	*Sac*I	Construction of pRAB11-EsaE-his[1]
EsaG-for-bgl II	GCGCAGATCTAGGAGGTTTCTACTTCAACATGACATTTGAAGAGA	gDNA	*Bgl*II	Construction of pRAB11-EsaG-his[1] and pRAB11-EsaG-HA[1]
esaG-rev-SacI-HA	GCGAGCTCTTATGCATAATCTGGAACATCATATGGATATTCTTCTA GCTCTTTAATATATT	gDNA	*Sac*I	Construction of pRAB11-EsaG-HA[1]
esaG-rev-his-EcoRI	GCGAATTCTTAGTGGTGGTGGTGGTGGTGTTCTTCTAGCTCTTTAA TATATT	gDNA	*Eco*RI	Construction of pRAB11-EsaG-his[1]
esaD-kpnI-for-esxA-RBS	GAAAGGTACCAGGAGTTTCTACTTATGACAAAAGA TATTGAATATCTAAC	gDNA	*Kpn*I	Construction of pRAB11-EsaD(H528A)-his[1] and pRAB11-EsaD(H528A)-HA[1]
esaD-his-to-ala-for	CGATGATGGAGGTGCATTAATCGCTAGAATG	gDNA		Change of His to Ala codon at codon 528 of *esaD*[2]
esaD-his-to-ala-rev	CATTCTAGCGATTAATGCACCTCCATCATCG	gDNA		Change of His to Ala codon at codon 528 of *esaD*[2]
EsaD-his-rev-bgl II	GCGCAGATCTCTAGTGGTGGTGGTGGTGGTGCTTATTTAATATTCT TCTAATATTTCT	gDNA/ *esaD(H528A)*	*Bgl*II	Construction of pRAB11-EsaD(H528A)-his[1] and pRAB11-EsaD$_{421-614}$(H528A)-His[1]
EsaD-HA-rev-bgl II	GCGCAGATCTCTATGCATAATCTGGAACATCATATGGATACTTATTT AATATTCTTCTAATATTTCT	gDNA /*esaD(H528A)*	*Bgl*II	Construction of pRAB11-EsaD(H528A)-HA[1]

esaD(421-614aa)-kpnI-for-esxA-RBS	GAAAGGTACCAGGAGGTTTCTACTTATGACACATGGTCCAAAAGATAGTATGGTGAG	*esaD(H528A)*	*Kpn*I	Construction of pRAB11-EsaD$_{421-614}$(H528A)-His[1]
00268-00278A1	CATGGAGCTCGATTGTACAATC	gDNA	*Sac*I	Construction of pIMAY-saouhsc_00268-00278[3]
00268-00278A2	TGTCTGCTACATGTCATGCACCTATCCCTC	gDNA		Construction of pIMAY-saouhsc_00268-00278[3]
00268-00278B1	CATGACATGTAGCAGACATGTTATAAAAGACTGTG	gDNA		Construction of pIMAY-saouhsc_00268-00278[3]
00268-00278B2	GCGCGAGCTCCATCTATTTCAGTGTTAATTTAC	gDNA	*Sac*I	Construction of pIMAY-saouhsc_00268-00278[3]
00268-00278-out1	TATGTATTTTGCACCATTTAGC			Sequencing primer
00268-00278-out2	CGTTTAAATGTTTGACGCAAGA			Sequencing primer
00274-00278A1	CATGGAGCTCGTATTACATATAGGTGTGGGT	gDNA	*Sac*I	Construction of pIMAY-saouhsc_00274-00278[3]
00274-00278A2	TGTCTGCTACATGTTATCGCCCCTATGTGTTGC	gDNA		Construction of pIMAY-saouhsc_0027400278[3]
00274-00278B1	GATAACATGTAGCAGACATGTTATAAAAGACTGTG	gDNA		Construction of pIMAY-saouhsc_00274-00278[3]
00274-00278B2	GCGCGGTACCCATCTATTTCAGTGTTAATTTAC	gDNA	*Kpn*I	Construction of pIMAY-saouhsc_00274-00278[3]
00274-00278-out1	GGAGCGTTTGCTTTTGTTGTAA	gDNA		Sequencing primer
0266A1	AGGATCCAGTGCTAATAAGGACAGTG	gDNA	*Bam*HI	Construction of pIMAY-esaE[3]
0266A2	CTCTGCTTTATCTTTCATCATGGGTTCAC	gDNA		Construction of pIMAY-esaE[3]
0266B1	ATGAAAGATAAAGCAGAGGAGTAATGACG	gDNA		Construction of pIMAY-esaE[3]
0266B2	TTCGGATCCAATTGAGATGCATAATC	gDNA	*Bam*HI	Construction of pIMAY-esaE[3]

0268A1	AGAAGGATCCAATGATTGGGACTTTAAAC	gDNA	*Bam*HI	Construction of pIMAY-esaD[3] and pIMAY-esaDG[3]
0268A2	CTTATTTAATTTTGTCATGTCATGCACC	gDNA		Construction of pIMAY-esaD[3] and pIMAY-esaDG[3]
0268B1	ATGACAAAATTAAATAAGTAGAGGTGCC	gDNA		Construction of pIMAY-esaD[3]
0268B2	TGAGGATCCACTTTACGGTATCTATTG	gDNA	*Bam*HI	Construction of pIMAY-esaD[3]
0269A1	GGTTGAATTCGGAGAACACTATGC	gDNA	*Eco*RI	Construction of pIMAY-esaG[3]
0269A2	TTATTCTTCAAATGTCATGTTGGCACCTC	gDNA		Construction of pIMAY-esaD[3]
0269B1	ATGACATTTGAAGAATAAACTATCTTAATG	gDNA		Construction of pIMAY-esaD[3] and pIMAY-esaDG[3]
0269B2	AAAGGATCCATGTTGCAAATACTGCG	gDNA	*Bam*HI	Construction of pIMAY-esaG[3] and pIMAY-esaD[3]
EsaG-out1	TGGGTCAAAACATAAAGCGTGC			Sequencing primer
EsaG-out2	ACGTGAACATTCCGCCAATTAC			Sequencing primer
RT-PCR-primer pair 1-for	ACATGGTCCAAAAGATAGTATGGTGAG	gDNA		RT-PCR
RT-PCR-primer pair 1-rev	GCACCTCTACTTATTTAATATTCTTC	gDNA		RT-PCR
RT-PCR-primer pair 2-for	GAAGAATATTAAATAAGTAGAGGTGC	gDNA		RT-PCR
RT-PCR-primer pair 2-rev	GTCATCATCTTCAGTGTTTAATTC	gDNA		RT-PCR
RT-PCR-primer pair 3-for	GATACTCTTCGAAAGCCAGGTGCAC	gDNA		RT-PCR

RT-PCR-primer pair 3-rev	GTCATCATCTTCAGTGTTTAATTC	gDNA		RT-PCR
pT25-EsxA-F	GCGCGGATCCCGCGATGATTAAAATGAGCCCGGAAG	Synthetic *esxA* gene sequence†	*Bam*HI	Construction of pT25-EsxA[4]
pT25-EsxA-R	GCGCGGTACCTTACTGCAGGCCAAAGTTGTTGCTCAGC	Synthetic *esxA* gene sequence†	*Kpn*I	Construction of pT25-EsxA[4]
pT25-EsxB-F	GCGCGGATCCCGGGCGGCTATAAAGGCATTAAAGC	Synthetic *esxB* gene sequence†	*Bam*HI	Construction of pT25-EsxB[4]
pT25-EsxB-R	GCGCGGTACCTTACGGGTTCACGCGATCCAGGCCCTG	Synthetic *esxB* gene sequence†	*Kpn*I	Construction of pT25-EsxB[4]
pT25-EsaB-F	GCGCGGATCCCAATCAGCACGTAAAAGTAACATTTG	Synthetic *esaB* gene sequence†	*Bam*HI	Construction of pT25-EsaB[4]
pT25-EsaB-R	GCGCGGTACCTTACAGCAGCAGTTTCAGAATATCGCCATCC	Synthetic *esaB* gene sequence†	*Kpn*I	Construction of pT25-EsaB[4]
pT25-EsaC-F	GCGCGGATCCCAACTTTAACGATATTGAAACGATG	Synthetic *esxC* gene sequence†	*Bam*HI	Construction of pT25-EsxC[4]
pT25-EsaC-R	GCGCGGTACCTTAGTTCATCGCTTTGTTAAAATATTCG	Synthetic *esxC* gene sequence†	*Kpn*I	Construction of pT25-EsxC[4]
T25esaD-F	GCGCGGATCCCACAAAAGATATTGAATATCT	*esaD(H528A)*	*Bam*HI	Construction of pT25-EsaD(H528A)[4]
T25esaD-R	GCGCGGTACCATTACTTATTTAATATTCTTCTAAT	*esaD(H528A)*	*Kpn*I	Construction of pT25-EsaD(H528A)[4]
T25esaE-F	GCGCGGATCCCAAAGATGTTAAGCGAATAGAT	gDNA	*Bam*HI	Construction of pT25-EsaE[4]
T25esaE-R	GCGCGGTACCATTACTCCTCTGCTTTATTAATAT	gDNA	*Kpn*I	Construction of pT25-EsaE[4]
T25esaF-F	GCGCGGATCCCACGTTGAGTGGAAAAATTAGTG	gDNA	*Bam*HI	Construction of pT25-EsxD[4]
T25esaF-R	GCGCGGTACCATTATCCCTCAATATTATAGT	gDNA	*Kpn*I	Construction of pT25-EsxD[4]
T25esaG-F	GCGCGGATCCCACATTTGAAGAGAAGCTTAG	gDNA	*Bam*HI	Construction of pT25-EsaG[4]

T25esaG-R	GCGCGGTACCATTATTCTTCTAGCTCTTTAATA	gDNA	*Kpn*I	Construction of pT25-EsaG[4]
T25esaD-421-614-F	GCGCGGATCCCACACATGGTCCAAAAGATAG	*esaD(H528A)*	*Kpn*I	Construction of pT25-EsaD$_{421-614}$(H528A)[4]
T18esaD-F	GCGCGGGCCCCACAAAAGATATTGAATATCT	*esaD(H528A)*	*Apa*I	Construction of pT18-EsaD(H528A)[5]
T18esaD-R	GCGCCTCGAGGGCTTATTTAATATTCTTCTAAT	*esaD(H528A)*	*Xho*I	Construction of pT18 EsaD(H528A)[5]
T18esaE-F	GCGCGGGCCCCAAAGATGTTAAGCGAATAGAT	gDNA	*Apa*I	Construction of pT18-EsaE[5]
T18esaE-R	GCGCCTCGAGGGCTCCTCTGCTTTATTAATAT	gDNA	*Xho*I	Construction of pT18-EsaE[5]
T18esaG-F	GCGCGGGCCCCACATTTGAAGAGAAGCTTAG	gDNA	*Apa*I	Construction of pT18-EsaG[5]
T18esaG-R	GCGCCTCGAGGGTTCTTCTAGCTCTTTAATA	gDNA	*Xho*I	Construction of pT18-EsaG[5]
T18esaD-1-420-R	GCGCCTCGAGGGCACGTGATTTTGTGGTGTAACAG	gDNA	*Xho*I	Construction of pT18-esaD$_{1-420}$[5]
pQE70-esaE-for	GCGAGCATGCATGAAAGACGTTAAACGTATC	Synthetic *esaE* gene sequence*	*Sph*I	Construction of pQE70-EsaE-HA-EsaG-his
pQE70-esaEG-overlapping-for	GAAATTAACCATGACCAAAAACAAATAATATTAAAGAGGAGAAATTAACCATGACCTTCGAAGAAAAACTGTC	Synthetic *esaEG* gene sequence*		Construction of pQE70-EsaE-HA-EsaG-his
pQE70-esaEG-overlapping-rev	CATATGATGTTCCAGATTATGCATAATGGATCCATTAAAGAGGAGAAATTAACCATGACCAAAAACAAATAAT	Synthetic *esaEG* gene sequence*		Construction of pQE70-EsaE-HA-EsaG-his
EsaG-for-SphI	GCGAGCATGCCCTTCGAAGAAAAACTGTCTAAAATC	Synthetic *esaG* gene sequence*	*Sph*I	Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his[6]
EsaG-overlapping-for	GTACATCAAAGAA CTGGAATAATAA ATTAAAGAGG AGAAATTAACC ATGACCTTCGAAGAAAAACTG	Synthetic *esaG* gene sequence*		Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his[6]
EsaG-overlapping-rev	CAGTTTTTCTTCGAAGGTCATGGTTAATTTCTCCTCTTTAATTTATTATTCCAGTTCTTTGATGTAC	Synthetic *esaG* gene sequence*		Construction of pQE70-EsaG-EsaG-EsaD421-614-his and

				pQE70-EsaG-EsaG-EsaD421-614(H528A)-his^{6}
EsaG-rev-apaI-bamHI	GCGAGGATCCGGGCCCTTATTATTCCAGTTCTTTGATGTACTGTTCG	Synthetic *esaG* gene sequence*	*Apa*I/*BamH*I	Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his^{6}
EsaDnu-for-ApaI	GCGA GGGCCC ATTAAAGAGGAGAAATTAACCATGCACGGTCCGAAAGACTCTATGGTTC	Synthetic *esaD* gene sequence*	*Apa*I	Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his^{6}
EsaDnu-rev-BglII	GCGAAGATCT TTTGTTCAGGATACGACGGATGTTACG	Synthetic *esaD* gene sequence*	*Bgl*II	Construction of pQE70-EsaG-EsaG-EsaD421-614-his and pQE70-EsaG-EsaG-EsaD421-614(H528A)-his^{6}
pQE70-esaD-qc-F	ACGACGACGGTGGTGCACTGATCGCGCGTATGTTCGGT	Synthetic *esaD* gene sequence*		Construction of pQE70-EsaE-HA-EsaD(H528A)-Myc-EsaG-his^{7}
pQE70-esaD-qc-R	ACCGAACATACGCGCGATCAGTGCACCACCGTCGTCGT	Synthetic *esaD* gene sequence*		Construction of pQE70-EsaE-HA-EsaD(H528A)-Myc-EsaG-his^{7}
pQE70-esaG-rev	GCGCAGATCTTTCCAGTTCTTTGATGTACTG	Synthetic *esaG* gene sequence*	*Bgl*II	Construction of pQE70-EsaE-HA-EsaG-his
pT7.5-esaD--for	AGTCTAGAAAGCTTTACTATAATATTGAGG	gDNA	*Xba*I	Construction of pT7.5esaD and pT7.5esaDG
pT7.5-esaD-xhoI-rev	CTACTCGAGCTTATTTAATATTCTTCTAAT	gDNA	*Xho*I	Construction of pT7.5esaD(V584Y)
pT7.5-esaD/G- xhoI-rev	GCCTCGAGTTCTTCTAGCTCTTTAATATATT	gDNA	*Xho*I	Construction of pT7.5esaDG
Intctrl1	CCAACTGCTGAAGTACCATTAACG			Checking chromosomal *ermC* integration
Intctrl2	GTACCTGCTATAAACAACGCGCAC			Checking chromosomal *ermC* integration

Restriction enzyme sequences are shown in underline.

[1]*esaE-His*, *esaE-HA* and *esaG-HA* were cloned as *Bgl*II-*Sac*I fragments, *esaG-His* was cloned as a *Bgl*II-*Eco*RI fragment and *esaD(H528A)-His, esaD(H528A)-HA* and *esaDNuc(aa 421-615; H528A)-His* were cloned as *Kpn*I-*Bgl*II fragments into similarly digested pRAB11. In each case the forward primer incorporated the *esxA* ribosome binding site (shown in red) to initiate translation.

[2]*esaD(H528A)* was generated by overlapping PCR generating DNA covering the N-terminus of *esaD* to just beyond codon 528 (amplified with primers esaD-kpnI-for-esxA-RBS and esaD-his-to-ala-rev introducing the H528A substitution) and from just prior to codon 258 to the end of *esaD* (amplified with primers esaD-his-to-ala-for and EsaD-his-rev-bgl II inducing H528A substitution). Subsequently the two fragments were spliced together by overlap PCR using primers esaD-kpnI-for-esxA-RBS and EsaD-his-rev-bgl II.

[3]The two flanking regions, upstream and downstream of the gene to be deleted including the first three and last three codons, were separately amplified with the A1/A2 and B1/B2 primer pairs. Next, overlapping PCR was performed with the A1 and B2 primers using the amplified flanking regions as template and the product was subsequently cloned into PIMAY (as a *Sac*I fragment for the *saouhsc_00268-saouhsc_00278* deletion, a *Sac*I-*Kpn*I fragment for the *saouhsc_00274-saouhsc_00278* deletion, a *Bam*HI fragment for the *esaD, esaDG* and *esaE* deletions and an *Eco*RI-*Bam*HI fragment for the *esaG* deletion). The primers out1 and out2 were using for sequencing to check the deletions, as shown schematically below.

[4]*esxA*, *esxB*, *esxC*, *esaB*, *esaD(H528A)*, *esaD*$_{421-614}$*(H528A)*, *esaE*, *esxD* and *esaG* were each cloned as *Bam*HI-*Kpn*I fragments into similarly digested pT25.

[5]*esaD(H528A)*, *esaD*$_{1-420}$, *esaE*, and *esaG,* were each cloned as *Apa*I-*Xho*I fragments into similarly digested pT18.

[†]A 10,651bp synthetic DNA sequence coding for EsxA, EsaA, EssA, EssB, EssC EsxC, EsxB from RN6390, codon optimised for expression in *E. coli* was used as template. The sequence of this synthetic DNA is given in Fig S12.

[6]Two copies of esaG were amplified, one with primer pair EsaG-for-SphI and EsaG-overlapping-for and the second with primer pair EsaG-overlapping-rev and EsaG-rev-apaI-bamHI and cloned into pQE70 by three way ligation as an *Sph*I-*Bam*HI fragment. DNA encoding EsaD421-614 or EsaD421-614(H528A) was subsequently cloned into this as an *Apa*I-*Bgl*II fragment.

*A 3,125bp synthetic sequence coding for EsaE, EsaD(H528A) and EsaG. Sequence given in Fig S13. [7]The (H528A) substitution of EsaD was introduced into this construct by Quickchange using the indicated primers.

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